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(57) Abstract

Methods are described for identifying inhibitors of the accelerated ubiquitin conjugation that occurs in disease states involving muscle wasting. Methods are also described for inhibiting the loss of muscle mass in such disease states by the use of inhibitors of key components of the N-end rule pathway for protein ubiquitination.

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METHODS OF INHIBITING PROTEIN DEGRADATION TO COMBAT MUSCLE WASTING

BACKGROUND OF THE INVENTION

1. Field of the Invention

The invention relates in general to inhibition of protein degradation, and in particular to inhibition of the increased or accelerated protein degradation as occurs in skeletal muscle in many diseases and results in muscle wasting.

2. Description of Related Art

Proteins in mammalian cells are continually being degraded to amino acids and replaced by protein synthesis. The bulk of proteins in the cytosol and nucleus of mammalian cells are degraded by a soluble proteolytic pathway that requires ATP. This process is not only responsible for the selective degradation of highly abnormal proteins and short-lived regulatory proteins, but in catabolic states it also plays a role in the breakdown of the longer-lived myofibrillar proteins. Most protein substrates degraded in this ATP-dependent pathway are first marked by covalent conjugation to the small protein cofactor, ubiquitin (Hershko and Ciechanover, 1992, Ann. Rev. Biochem. 61:761; Ciechanover, 1994, Cell 79:13).

As used herein, "ubiquitination" generally refers to conjugation of ubiquitin to a target protein or to the proteins to which it normally becomes conjugated in the eukaryotic cell. The ubiquitination of protein substrates in eukaryotic cells involves the following steps: activation of the carboxyl end of ubiquitin in an ATP-requiring reaction catalyzed by the ubiquitin activating enzyme E1, transfer of activated ubiquitin to an E2 carrier protein, and transfer of ubiquitin from E2 to the ε-amino group of a lysine in the protein substrate. The final coupling step is generally catalyzed by a third ubiquitination enzyme, an E3 ligase. Additional ubiquitin molecules are linked in turn to a specific lysine of the first ubiquitin to form long chains of ubiquitin attached to the protein substrate, which is then rapidly degraded by the 26S proteasome, a 2000 kD complex containing multiple peptidase activities. The ubiquitin conjugation process is generally believed to be the rate-limiting step in the ATP-dependent protein degradative pathway (Hershko and Ciechanover, 1992, supra; Ciechanover, 1994, supra). Each organism contains one or two ubiquitin activating E1 enzymes, which are highly conserved in evolution. Selectivity in the pathway is provided by the E2 and E3 enzymes, which operate in pairs in the ubiquitination of different types of proteins. Specific E2 and E3 enzymes have been shown to catalyze ubiquitin conjugation to the cyclin protein that regulates mitosis (King et al. 1995, Cell 81:279), while a distinct E2 and E3

enzyme pair catalyzes conjugation of ubiquitin to the tumor suppressor, p53 (Scheffner et al., 1993, Cell 75:495).

A number of structural features operate to target proteins for ubiquitination and, thereby, for degradation by the proteasome. The simplest such structural feature, and the first to be elucidated, relates the stability of a protein to its amino terminal residue. This effect, designated the "N-end rule pathway", is based on the finding that certain of the 20 amino acids, if present in the amino terminal position, can lead to rapid degradation of an otherwise stable protein, as in the case of β-galactosidase protein. Species of this protein or other proteins that contain abnormal N-termini have been generated by genetic engineering. Amino acids which lead to rapid degradation have been termed "destabilizing" while ones that do not cause rapid degradation are termed "stabilizing". For example, Bachmair et al., 1986, Science 234:179 hereby incorporated by reference in its entirety lists the half lives of amino acid X-βgalactosidase complexes and indicates that at least the following amino acids may be catagorized as being destabilizing to various degrees: isoleucine (Ile), glutamine (Gln), glutamate (Glu), tyrosine (Tyr), leucine (Leu), phenylalanine (Phe), aspartate (Asp), lysine (Lys), arginine (Arg), asparagine (Asn), histidine (His) and tryptophan (Trp).

All proteins as normally synthesized begin with the stabilizing residue, methionine. According to the N-end rule pathway, targeting of a proteolytic substrate for degradation requires the generation of a residue at the amino terminus of the substrate, which is susceptible to interaction with an N-end-recognizing enzyme prior to ubiquitination of the substrate by either the same N-end-recognizing enzyme alone or in concert with other ubiquitin-conjugating enzymes.

As an example, it was determined that the specific recognition element of the N-end rule pathway in yeast is an E3 ligase, UBR1 (Bartel et al, 1990, EMBO J. 9:3179) and in reticulocytes, is the E3α ligase. UBR1 and the mammalian homolog each contain binding sites for both N-terminal bulky hydrophobic residues (Phe, Leu, Trp, Tyr) and basic residues (Arg, Lys, His). In addition, mammalian cells contain a second N-end rule ubiquitin-protein ligase, E3β, which recognizes small uncharged residues (Ala, Ser, Thr). Both E3α and E3β function in concert with the E2 enzyme, $E2_{14k}$.

Chemical inhibitors of E3α are known (Hershko and Ciechanover, 1992, supra). For example, dipeptides, amino acid hydroxamates, and amino acid methyl esters with a basic N-terminal residue inhibit the ubiquitin conjugation and degradation of proteins such as lysozyme which has a basic N-termini, while derivatives of dipeptides, amino acid hydroxamates, and amino acid methyl esters with bulky hydrophobic N-terminal residues inhibit the ubiquitin conjugation and degradation of proteins such as β-lactoglobulin with hydrophobic N-termini. Similarly, the

degradation of proteins with small uncharged N-termini (Ala, Ser, Thr) can be inhibited by dipeptides with small uncharged N-terminal residues, presumably by inhibition of E3β (Gonda et al., 1989, J. Biol. Chem. 264:16700).

Individual proteins are degraded at different rates, and the average rate of protein turnover differs from tissue to tissue. Normally, the rates of protein synthesis and protein degradation in each cell are precisely balanced; even a small increase in net protein degradation can result in profound loss of body mass, such as occurs in many conditions or disease states such as hyperthyroidism, AIDS- or cancer-related cachexia, sepsis, metabolic acidosis, spinal injury, systemic infections, muscle denervation or disuse atrophy and the like and is a result of excessive or accelerated protein breakdown in muscle tissue. Studies in animal models that mimic these human disease states indicate that the enhanced protein breakdown is primarily due to the activation of the ubiquitin-proteasome pathway (Goldberg, U.S. Patent No. 5,340,736 hereby incorporated by reference in its entirety).

The precise mechanism for activation of this pathway in disease states and specifically which enzymes contribute to the accelerated degradation of specific proteins leading to muscle wasting, however, is not understood. Greater understanding of the mechanism(s) that accelerate muscle protein turnover due to the activation of the ubiquitin-proteasome pathway should lead to the development of therapies that suppress accelerated proteolysis in catabolic states.

Studies of the mechanisms causing loss of muscle mass during fasting, sepsis, metabolic acidosis, cancer and/or denervation atrophy demonstrate that the loss of protein is primarily due to enhanced proteolysis, especially of myofibrillar proteins. The increase in proteolysis was not inhibited when the atrophying muscles were incubated in vitro with agents that blocked the activity of lysosomes and calcium-activated proteases. However, when inhibitors of ATP production were added, muscle protein degradation decreased to the level measured in control muscles (Wing and Goldberg, 1993, Amer. Jour. Physiol. 264:E668; Mitch et al., 1994, Jour. Clin. Invest. 93:2127). Using this approach, the ATP-dependent degradative process could be measured in muscles under defined conditions in vitro. Fasting or denervation stimulated the ATP-dependent process 2-3 fold in muscles and this response accounted for the overall increase in proteolysis.

Although consistent with activation of the ATP-ubiquitin-proteasome pathway, such experiments using metabolic inhibitors can be misleading. More definitive evidence that the ubiquitin-proteasome pathway is stimulated by denervation or fasting comes via the observation that levels of ubiquitin-protein conjugates were increased in muscles at the same time that protein degradation was maximally accelerated; when the fasted rats were refed, these critical intermediates

decreased along with the fall in proteolysis (Wing et al., 1995, Biochem. Jour. 307:639). The accumulation of ubiquitin-conjugated proteins is clear evidence that ubiquitination of cell proteins and the flux of substrates through this pathway are accelerated in the atrophying muscle.

In addition, it has been observed that the levels of mRNAs encoding ubiquitin and several different subunits of the 20S proteasome increased during fasting and denervation atrophy (Medina et al., 1995, Biochem. Jour. 397:631; Medina et al., 1991, Biomed. Biochim. Acta 50:347). These mRNA levels increased despite a fall in the total RNA content in muscle, and they decreased rapidly upon refeeding of the fasted rats despite an increase in total RNA. In mammals, there are several genes encoding ubiquitin. However, in catabolic states, only the mRNA encoded by the polyubiquitin gene increases in muscle. This gene is unusual in that it contains several ubiquitin genes in tandem; it is translated as a polyprotein, which is then cleaved to yield multiple ubiquitin molecules (Nenoi et al., 1994, Biochem. Biophys. Res. Comm. 1204:271). Fasting also leads to an increase in $E2_{14k}$ gene expression (Wing and Banville, 1994, Am. J. Physiol. 267:E39).

MG 132 and MG 115 are peptide aldehydes that have been shown to inhibit several peptidase activities of the 20S proteasome (Palombella et al., Cell, 1994, 78:773; Rock et al., 1994, Cell, 78:761). In isolated muscles from normal rats, MG 132 and MG 115 cause a marked reduction in overall protein degradation. An even greater decrease in proteolysis is produced in muscles treated with thyroid hormone or in muscles from denervated rats, rats with uremia, or thyroid hormone-treated rats. These data provide further evidence that the ubiquitin-proteasome pathway is responsible for the enhanced proteolysis observed in such conditions, and suggest that inhibitors of proteasome or protein ubiquitination would be useful to combat muscle wasting.

However, the data provide no information about other enzymes of the pathway altered in these disease states and that may be targets for inhibition in order to reduce or combat the loss of muscle mass. In fact, while the N-end rule pathway has been extensively studied and characterized, its physiological relevance or significance has remained obscure. In fact, a yeast UBR1 null mutant grows normally and exhibits only a mild phenotype (Bartel et al., 1990, EMBO J., 9:3179). Most importantly, the great majority of intracellular proteins, such as those in muscle tissue would not be expected to be substrates for the N-end rule pathway because the great majority of cell proteins begin with methionine (which has been identified as a stabilizing residue) or have an acetylated residue at the N termini and post-translational cleavage of the initiator methionine residue by methionine aminopeptidase occurs only if the second residue is one which will produce a stable amino acid, i.e. a stabilizing residue. Accordingly, no destabilizing amino acid would be predicted for muscle tissue which would make it a substrate for the N-end rule pathway.

Accordingly, there is a need in the art for effective treatment of muscle wasting through the inhibition of enzymes responsible for acceleration of the ubiquitin-proteasome degradative pathway.

One object of the invention is to provide methods for identifying inhibitors of protein degradation which occurs in muscle wasting.

Another object of the invention is to provide models that mimic protein degradation which occurs in muscle wasting in order to screen for inhibitors of such degradation.

Yet another object of the invention is to identify enzymes that are responsible for acceleration of ubiquitin conjugation which occurs in diseases involving muscle wasting, and to identify inhibitors of these enzymes.

Yet another object of the invention is to suppress the rate of ubiquitination in atrophying muscle to the level of ubiquitination in normal muscle.

Yet another object of the invention is to inhibit muscle wasting by interfering with key enzymes involved in ubiquitin conjugation of muscle protein.

SUMMARY OF THE INVENTION

Embodiments of the present invention are based, in part, on the discovery that the acceleration of ubiquitin conjugation that occurs in disease states involving abnormally high levels of muscle wasting is due to changes in the activity or levels of key components that are involved in protein degradation via the N-end rule pathway.

Embodiments of the present invention are also based, in part, on the discovery that the N-end rule pathway for protein ubiquitination is a major pathway for protein degradation in skeletal muscle extracts, and that during muscle atrophy in septic, tumor-bearing, diabetic, and hyperthyroid animals the activity of this pathway increases as a result of increases in the amounts or activities of certain components of the N-end rule pathway for protein ubiquitination. These components function, either alone or in concert, by activating ubiquitin, by directly recognizing the muscle protein as having a destabilizing amino terminal residue, by modifying the muscle protein so that it can be recognized by an ubiquitination enzyme as having a destabilizing amino terminal residue, or to facilitate ubiquitination of the muscle protein once it has been recognized as having a destabilizing amino terminal residue.

A single E1 ubiquitin activating enzyme, or at most two, exist in eukaryotes. By contrast, multiple E2's and E3's exist in eukaryotes, the corresponding molecules differing primarily in their substrate specificities. As used herein, the enzyme E1 refers to a eukaryotic ubiquitin activating enzyme which functions to catalyze the activation of ubiquitin in an ATP-requiring reaction of the

ubiquitin-proteasome pathway. The enzyme E2 refers to a eukaryotic enzyme which functions as a carrier protein in the transfer of activated ubiquitin to the protein substrate in a reaction catalyzed by the enzyme E3. The enzyme E3 refers to a eukaryotic enzyme that functions as a ubiquitin protein ligase, in which ubiquitin is ligated to the ε-amino group of the substrate protein. E2 and E3 enzymes are further referred to as ubiquitin-conjugating enzymes. N-terminal protein modifying enzymes refer to enzymes which modify the N-terminal residue of a protein so that it may be recognized by an ubiquitin-conjugating enzyme.

Embodiments of the present invention are further based, in part, on the discovery that inhibition of one or more components of the N-end rule pathway for protein ubiquitination, including specific ubiquitin-conjugating enzymes and N-terminal protein modifying enzymes, prevents the accelerated ubiquitin conjugation that otherwise occurs in septic, cancer-bearing, diabetic, and/or hyperthyroid animals in which accelerated muscle proteolysis is observed. The invention thus recognizes for the first time that the N-end rule pathway is not only a major degradative pathway, but also appears to be responsible for most of the increased proteolysis in atrophying muscle. As previously stated, the great majority of intracellular proteins, such as those in muscle tissue would not be expected to be substrates for the N-end rule pathway because the great majority of cell proteins begin with methionine (which has been identified as a stabilizing residue) or have an acetylated residue at the N termini and post-translational cleavage of the initiator methionine residue by methionine aminopeptidase occurs only if the second residue is one which will produce a stable amino acid, i.e. a stabilizing residue. Accordingly, no destabilizing amino acid would be predicted for muscle tissue which would make it a substrate for the N-end rule pathway.

Embodiments of the present invention therefore encompass methods of inhibiting muscle wasting in a mammal resulting from degradation of muscle protein via the N-end rule pathway by interfering with the function of one or more components of the N-end rule pathway for protein ubiquitination so as to suppress ubiquitination of the muscle protein resulting in reduced muscle wasting.

The function of the components of the N-end rule pathway for protein ubiquitination, whether the components are ubiquitin activating enzymes, ubiquitin-conjugating enzymes or N-terminal protein modifying enzymes, can be interfered with by inhibiting the component's biological activity whether by means of a direct inhibiting agent or by reducing the amount of the component relative to the amount of component present during muscle wasting. A decrease in the amount of the component may occur at the level of expression of the gene encoding the enzyme, or at the level

of translation of the mRNA encoding the enzyme, or at the level of the stability of the enzyme (i.e., by rendering the enzyme less stable).

Embodiments of the invention further encompass methods of inhibiting degradation in cultured muscle cells or in muscle cell extracts of muscle protein by contacting the culture or extract with an inhibitor that interferes with the function of one or more components of the N-end rule pathway for protein ubiquitination so as to suppress ubiquitination of the muscle protein. Embodiments of the invention still further encompass methods for the screening of inhibitors of muscle wasting in test samples or in cell free or cell based assay systems. Kits for carrying out the above described methods are also included within embodiments of the present invention.

Further features and advantages of certain embodiments of the present invention will become more fully apparent in the following description of the embodiments and drawings thereof, and from the claims.

BRIEF DESCRIPTION OF DRAWINGS

In the course of the detailed description of certain preferred embodiments to follow, reference will be made to the attached drawings, in which,

Fig. 1 is a graph demonstrating that the rate of ubiquitin-conjugation to cell proteins is lower in muscle extracts of hypophysectomized rats than in muscle extracts of normal rats.

Fig. 2 is a computer generated autoradiogram showing that an E3α inhibitor, arginine methyl ester, reduces the level of ubiquitin conjugation in normal rat muscle extracts (C) to levels comparable to those in muscle extracts from hypophysectomized rats (H). Alanine methyl ester, which does not inhibit E3α, has no effect on ubiquitin conjugation.

Fig. 3 is a computer generated autoradiogram showing that the rate of ubiquitin-conjugation to muscle proteins is reduced in thyroidectomized rats (Tx) compared to normal rats (C). Treatment of thyroidectomized rats for 7 days with high doses of triiodothyronine (T3), which enhances proteolysis to normal levels, stimulates ubiquitin-conjugation.

Fig. 4 is a computer generated autoradiogram showing the that rate of ubiquitin conjugation to muscle proteins is reduced in hypophysectomized rats (H) compared to normal rats (C). Treatment of hypophysectomized rats with doses of triiodothyronine (T3), which enhances proteolysis to normal levels, stimulates ubiquitin-conjugation.

Fig. 5 is a bar graph showing the results of an experiment in which muscle extracts from control ("C"), thyroidectomized ("TX) and T3-treated thyroidectomized ("T3") rats were prepared

and the rate of ubiquitination to soluble proteins was assayed in the presence or absence of arginine-methyl ester (1mM) or alanine-methyl ester (1mM).

Fig. 6 is a computer generated autoradiogram showing that sepsis induced by cecal ligation and puncture leads to enhanced rates of ubiquitin-conjugation to muscle protein in rat muscle extracts.

Fig. 7 is a computer generated autoradiogram showing that two inhibitors of E3α, arginine methyl ester and leucine methyl ester, each reduce the enhanced ubiquitin-conjugation seen in muscle extracts from septic animals (S) toward levels comparable to those seen in normal rats (C).

Fig. 8 is a graph of tyrosine generated versus time upon the addition of ATP and ubiquitin to soluble proteins of rabbit muscle extract demonstrating that ATP and ubiquitin stimulate the degradation of soluble proteins in rabbit skeletal muscle extract.

Fig. 9 is a graph showing that the rates of ubiquitination of endogenous muscle proteins for cachectic rats implanted with YAH are increased relative to rates of ubiquitination in muscle extracts from control rats.

Fig. 10 is a computer generated autoradiogram showing results of measuring ubiquitin conjugation over time in muscle extracts from control and YAH bearing rats.

Fig. 11 is a graph showing the rate of ubiquitin conjugation to endogenous soluble proteins over time in mice bearing colon-26 tumor, control mice and mice after tumor removal.

Fig. 12 is a graph showing the rate of ubiquitin conjugation to ^{125}I-lysozyme over time in mice bearing colon-26 tumor, control mice and mice after tumor removal.

Fig. 13 is a graph representing the amount of ubiquitination measured in muscle extracts from control and cachectic rats in the presence or absence of known E3α inhibitors.

Fig. 14 is several bar graphs showing ubiquitination in (A)septic rats "S", (B) rats bearing YAH tumors "Y", (C) hypophysectomized rats "H", and (D) thyroidectomized rats "Tx" versus control rats "C" upon introduction of arginine methyl ester (Arg ME), leucine methyl ester (Leu ME) and alanine methyl ester (Ala ME).

Fig. 15 is computer generated autoradiogram showing ubiquitin conjugation of ^{125}I-lysozyme over time in control rats and septic rats.

Fig. 16 is computer generated autoradiogram showing ubiquitin conjugation of ^{125}I-lysozyme over time in control rats and rats bearing the YAH tumor.

Fig. 17 is computer generated autoradiogram showing ubiquitin conjugation of ^{125}I-lysozyme over time in control rats and thyroidectomized rats.

Fig. 18 is computer generated autoradiogram showing ubiquitin conjugation of ^{125}I-lysozyme over time in control rats and hypophysectomized rats.

Fig. 19 shows several computer generated autoradiograms showing ^{125}I-ubiquitin conjugation to soluble muscle proteins in control and various pathologic states.

Fig. 20 shows several computer generated autoradiograms showing the results of Western blots of E1 and $E2_{14k}$ and a thiolester assay of muscle from rats rendered diabetic.

Fig. 21 shows several computer generated autoradiograms showing the results of Western blots of E1 and $E2_{14k}$ and a thiolester assay of muscle from rats with chronic renal failure.

Fig. 22 shows several computer generated autoradiograms showing the results of Western blots of E1 and $E2_{14k}$ from rats bearing Yoshida Ascites Heptoma (YAH).

Fig. 23 shows several computer generated autoradiograms showing the results of Western blots of E1 and $E2_{14k}$ and a thiolester assay of muscle from hypophysectomized rats.

The content of each reference referred to herein is hereby incorporated by reference in its entirety.

DETAILED DESCRIPTION OF CERTAIN PREFERRED EMBODIMENTS

The principles of the present invention may be applied with particular advantage to obtain methods for inhibiting muscle wasting by interfering with the function of one or more components of the N-end rule pathway for protein ubiquitination so as to suppress ubiquitination of muscle protein thereby reducing muscle wasting.

For the first time, it is herein demonstrated that ubiquitin-conjugation via the N-end rule pathway is accelerated in atrophying muscles and suppressed in muscles from animals in which protein degradation is reduced. It is also demonstrated for the first time that the accelerated ubiquitination that occurs in muscle wasting can be suppressed or reversed with inhibitors of either N-terminal protein modifying enzymes or N-end rule ubiquitin-conjugating enzymes. Such inhibitors may interfere with the ability of the N-terminal protein modifying enzymes or N-end rule ubiquitin-conjugating enzymes to function and/or to either modify muscle proteins for recognition by ubiquitin-conjugating enzymes or to conjugate ubiquitin to the muscle protein.

In a preferred embodiment, the ubiquitin-conjugating enzyme active in the N-end rule pathway is an E2 or E3 enzyme, and more particularly is $E2_{14k}$ or E3α. In a further preferred embodiment, an inhibitor of the ubiquitin-conjugating enzyme is utilized to interfere with its activity. For example, an amino acid ester or dipeptide inhibitor of E3α or derivatives thereof may be used.

Embodiments of the present invention are also directed to the practice of the above method of inhibiting muscle wasting in a mammal afflicted with a muscle wasting disease. Examples of such muscle wasting diseases include hyperthyroidism, AIDS- or cancer-related cachexia, sepsis, metabolic acidosis, spinal injury, systemic infections, denervation or disuse atrophy and the like. It is important to understand that other muscle wasting diseases are encompassed by the teachings of the present invention and would be readily identifiable to one skilled in the art based on the teachings presented herein.

Embodiments of the present invention are also directed to methods of inhibiting, in cultured muscle cells or in muscle cell extracts, degradation of muscle protein by contacting the culture or extract with an inhibitor that interferes with the function of one or more components of the N-end rule pathway for protein ubiquitination so as to suppress ubiquitination of the muscle protein thereby reducing muscle wasting.

Embodiments of the present invention are further directed to methods of screening for an inhibitor of muscle wasting by measuring ubiquitination in a cell-free assay system comprising: 1) a muscle extract containing one or more components of the N-end rule pathway for protein ubiquitination; 2) exogenous detectably labeled ubiquitin; and 3) a candidate inhibitor; wherein a decrease in ubiquitination in a sample containing the candidate inhibitor relative to that in a control sample without the inhibitor is indicative of inhibition by the candidate inhibitor.

Embodiments of the present invention are still further directed to methods of screening for an inhibitor of muscle wasting by measuring ubiquitination in a test sample comprising: 1) a muscle extract containing one or more components of the N-end rule pathway for protein ubiquitination which are from an atrophying muscle; 2) exogenous detectably labeled ubiquitin; and 3) a candidate inhibitor; wherein a decrease in ubiquitination in a sample containing the candidate inhibitor relative to that in a control sample without the inhibitor is indicative of inhibition by the candidate inhibitor.

Preferably, the method comprises comparing the levels of ubiquitination in the test sample and control sample with the level of ubiquitination in a standard sample, the standard sample comprising: 1) a muscle extract containing one or more components of the N-end rule pathway for protein ubiquitination from a normal muscle; and 2) exogenous detectably labeled ubiquitin; wherein inhibition of the enhanced ubiquitination in atrophying muscles relative to normal muscles can be determined.

In preferred embodiments, the muscle extract of the test samples is from a mammal afflicted with a condition that results in a catabolic state in muscle tissues of the mammal. Preferably, the mammal exhibits muscle wasting as a result of excessive protein degradation, as occurs in conditions

or diseases such as hyperthyroidism, AIDS- or cancer-related cachexia, sepsis, metabolic acidosis, spinal injury, systemic infections, denervation or disuse atrophy and the like.

To measure ubiquitination in the above assays, the ubiquitin conjugates formed can be separated from the remainder of the reaction mixture, and the amount of labeled ubiquitin that has been incorporated can be determined.

Embodiments of the present invention are even still further directed to methods of screening for an inhibitor of muscle wasting, the method comprising 1) incubation of a detectably labeled ubiquitination substrate and a candidate inhibitor with a cell-free muscle extract containing one or more components of the N-end rule pathway for protein ubiquitination; and 2) measuring proteolysis of the substrate; wherein a decrease in substrate degradation in the presence of the inhibitor relative to that in a control sample in the absence of the inhibitor is indicative of inhibition by the candidate inhibitor.

Preferably, the ubiquitination substrate is an N-end rule substrate. More preferably, the ubiquitination substrate is one of lysozyme, lactalbumin, or β-lactoglobulin. Alternatively, one can observe ubiquitin conjugation to muscle protein generally using ^{125}I-Ubiquitin or can measure degradation of total cell protein by measuring production of free tyrosine.

Preferably, the candidate inhibitor for the above-described assays is a dipeptide, amino acid hydroxamate, or amino acid methyl ester or any derivative thereof having an amino terminal residue which mimics the amino terminus of an N-end rule substrate protein.

In preferred embodiments of the above-described assays, the muscle extract contains the N-end rule ubiquitin-conjugating enzymes $E2_{14k}$ and E3α.

In other embodiments, the assay system may comprise, in place of the muscle extract, one or more purified components of the N-end rule pathway for protein ubiquitination such as an N-end rule ubiquitin conjugating enzyme, preferably, $E2_{14k}$ and E3α.

Still further embodiments of the present invention are directed to kits for carrying out the above-described inventive methods. The kits will include a muscle extract containing one or more components of the N-end rule pathway for protein ubiquitination such as an N-end rule ubiquitin-conjugating enzyme, exogenous detectably labeled ubiquitin, and packaging materials therefor. Alternatively, the kit may include a muscle extract containing one or more components of the N-end rule pathway for protein ubiquitination such as an N-end rule ubiquitin-conjugating enzyme, and a detectably labeled ubiquitination substrate, and packaging materials therefor. Each of the muscle extracts, and the exogenous ubiquitin or the ubiquitination substrate may be packaged together or separately.

Preferably, the muscle extract is from an atrophying muscle from, for example, a mammal afflicted with a condition that results in a catabolic state in muscle tissue of the mammal. The mammal thus exhibits an abnormally high level of muscle wasting as occurs in hyperthyroidism, cancer or sepsis. The kit may further include a standard muscle extract containing one or more components of the N-end rule pathway for protein ubiquitination such as an N-end rule ubiquitin-conjugating enzyme from a normal muscle.

In other embodiments of the above-described assays and assay kits, the test muscle extract is from a normal muscle and the standard muscle extract is from a mammal afflicted with a condition that results in a lower rate of catabolism in muscle tissue than the rate of catabolism in normal muscle tissue. Examples of such afflicted mammals include thyroidectomized or hypophysectomized mammals.

Even still further embodiments of the present invention are directed to cell-based assay methods of screening for an inhibitor of muscle wasting, wherein the cells are engineered to produce a protein that is subject to degradation via the N-end rule pathway. Preferably, the protein possesses a reporter activity that can be readily assayed, such that the level of the reporter activity is indicative of the amount of the protein present in the cell. Examples of proteins suitable for use in this assay include mutant forms of β-galactosidase that bear an amino acid such as leucine or arginine at the *N*-terminus, which renders the protein susceptible to degradation via the N-end rule pathway. Such proteins can be introduced into mammalian cells using standard methods and gene constructs similar to those described by Varshavsky and co-workers (Bachmair et al., 1986, Science, 234:179). Briefly, cells are transformed with a genetic construct encoding a ubiquitin—β-galactosidase fusion protein. The linear fusion protein undergoes rapid deubiquitination by cellular ubiquitin-C-terminal hydrolases to produce the β-galactosidase protein, which has an abnormal N-terminal residue and which can be assayed by methods known in the art.

The invention also encompasses a kit for screening for a candidate inhibitor of muscle wasting, the kit comprising cells containing a recombinant DNA construct encoding a protein susceptible to degradation via the N-end rule pathway, and packaging materials therefor. Preferably the DNA construct encodes a ubiquitin—protein fusion product. More preferably, the DNA construct encodes a ubiquitin—β-galactosidase fusion protein, most preferably one in which the N-terminal residue of β-galactosidase is arginine or leucine.

The following descriptions of experimental protocol are to be taken in conjunction with the experimental examples to follow.

ANIMAL MODELS OF MUSCLE WASTING

Described below are animal models in which the rate of muscle protein breakdown is abnormally high (i.e., septic or T3 treated animals) or low (i.e., thyroidectomized or hypophysectomized animals). These model animals were prepared as follows.

Hypophysectomized and thyroidectomized, as well as control (non-operated) CD male rats (as 70-90 gm at death), are purchased from Charles River Laboratory, Wilmington, Ma. To ensure the clearance of circulating hormones and proteins dependent of anterior pituitary hormones or thyroid hormones following hypophysectomy or thyroidectomy, rats are generally used for the experiments only after 15-30 days following the surgery. During this period, the weight of each animal is reported every day. T3 treatments begin by giving the animals a single subcutaneous injection daily. Thyroidectomized rats receive 20μg/100g body weight T3 hormone for 7 days and hypophysectomized animals receive daily 100μg T3/100g body weight for 4 days. The control rats are given a similar volume of saline. The body temperature and the weight of each animal is observed regularly during the thyroid hormone treatment.

Male rats are purchased from Charles River Laboratory, Wilmington, Ma. The standard method for inducing a fulminant type of sepsis in rats, called "cecal ligation and puncture" (CLP), is used. Briefly, under light anesthesia, a 3cm midline incision is made. The cecum is isolated, ligated in such a way so as to maintain the gut continuity, and is punctured in one place on the antimesenteric border with an 18 gauge needle. The needle is rotated at puncture site, and a small amount of fecal material is forced through the perforation to ensure that it is not sealed off. The punctured cecum is then returned to the peritoneal cavity, and the abdominal incision is closed. These procedures mimic peritonitis and sepsis in human patients.

Animals from each experimental group (such as control and hypophysectomized, control and hypophysectomized and thyroid treated, control and thyroidectomized and T3-treated or control and sepses) are sacrificed on the same day and extracts are prepared from these muscles in parallel under the identical conditions. Seven animals in each group are generally used for each experiment.

Muscles from the above-described animal models are then analyzed for rates of ubiquitin conjugation. First muscle tissue is homogenized, and myofibrils, membranes and proteasomes are removed by prolonged centrifugation, and subjected to ion-exchange chromatography to remove ubiquitin. The conjugation of exogenous radiolabeled ubiquitin to soluble cell proteins is then measured.

PREPARATION OF MUSCLE EXTRACTS

Leg muscles (soleus, quarter-diaphragm, atria and extensor digitorum longus, all together) are excised after the rats are killed by cervical dislocation. Muscles from each rat are then frozen separately in liquid nitrogen and then stored at -70°C until use.

To prepare extracts, frozen muscles from 3-6 rats from each group are pooled and 20% (w/v) muscle extracts are prepared as follows. Muscles are ground in a prechilled dounce all glass homogenizer. Approximately 3gm of muscles are suspended in ice-cold buffer (4ml per gm of tissue) containing 20mM Tris (pH 7.8), 1mM B-mercaptoethanol, 1% glycerol, 1mM EDTA and 1mM EGTA. Chymostatin and E-64 (final concentration 20μg/ml) are added during the extraction to block the chymase activity released from mast cells, lysosomal proteases and Ca^{2+}-dependent proteinases. Homogenates are centrifuged at 10,000xg for 30 minutes to remove myofibrils. The "crude extracts" are then prepared by centrifuging 20mM Tris (pH 7.6), 1mM DTT, 10mM magnesium acetate, 20mM potassium chloride. To remove endogenous ubiquitin, the extracts are then fractionated by chromatography on DEAE-cellulose (Fagan, J.M., Waxman, L. and Goldberg, A.L. (1987) Biochem J. 243, 335-343) into Fraction-II (F-II), the resin-bound material, which contains most of enzymes required for ubiquitin conjugation. The bound material is then eluted using 0.5 M NaCl. These fractions are then stored in small aliquots at -70°C in a buffer containing 20mM Tris pH 7.4, 1mM DTT, 10mM $MgCl_2$ and 20% glycerol until use. For degradation assays, the Fraction-II is then used directly.

For ubiquitination assays the Fraction-II is further centrifuged for 6-8 hours at 100,000g to remove the proteasome particles (in order to avoid degradation of the ubiquitin conjugates by the 26S proteasome complex or by the associated isopeptidases). Such extracts are good for ubiquitination assays for only up to 15 days.

MEASUREMENT OF UBIQUITIN CONJUGATION

Conjugation of ^{125}I-ubiquitin to soluble muscle proteins is measured as follows. The reaction mixture (25μl) contains 50mM Tris, pH 7.6, 5mM $MgCl_2$, 2mM DTT, 2mM ATPγS, 3μg of ^{125}I-ubiquitin, and 50μg of Fraction-II depleted of proteasomes. ATPγS is an ATP analog that supports ubiquitin conjugation but does not support subsequent degradation of the ubiquitinated proteins. Following the incubation at 37°C for 1 hour, the reaction is terminated by addition of sample buffer (25mM Tris pH 6.8, 20mg/ml SDS, 0.1mg/ml bromophenolblue, 5mM B-mercaptoethanol, 2mM EDTA and 10% glycerol). 12.5% Polyacrylamide-gel-electrophoresis in presence of SDS is then

performed as described by Laemmeli (Laemmli, U.K. (1970) Nature 227, 660-685). The gels are then dried and auto-radiographed.

The conjugation of ^{125}I-ubiquitin to soluble muscle proteins is then determined by comparing the formation of new, high molecular weight radio-labeled bands >8kDa, molecular weight of ^{125}I-ubiquitin) in the absence (control) or presence of soluble substrate proteins (Fraction-II depleted of proteasomes). Since ubiquitin conjugation is a time and energy dependent process, control reactions can be a reaction mixture incubated at time 0 or a reaction mixture incubated for 60 minutes in the absence of ATPγS.

To measure the activity of E3 in the extract, the conjugation of unlabeled ubiquitin and ^{125}I-labeled protein substrate such as ^{125}I-lysozyme or ^{125}I-ribonuclease-S-protein is measured. The reaction mixture contains 3μg of labeled ^{125}I-lysozyme and 10μg of unlabeled ubiquitin and 100μg of proteasome depleted Fraction-II.

Ubiquitin conjugation to the endogenous proteins in the extract and to certain model exogenous proteins is assayed using the above measurements of ubiquitin conjugation. This conjugation step is the rate-limiting step in protein breakdown in muscle wasting.

CANDIDATE INHIBITORS

"Inhibitor" refers to a molecule, e.g., a protein, peptide, dipeptide, or analog thereof, or a synthetic chemical that reduces muscle wasting or the level of protein degradation in a muscle cell. Inhibitors are identified according to any of the assays described herein, e.g., by measuring substrate degradation, ubiquitination levels, or reporter enzyme activity levels. For screening of candidate inhibitors, reaction mixtures also include a candidate inhibitor, for example, a dipeptide, amino acid methyl ester, or amino acid hydroxamate inhibitor. A candidate inhibitor may be present at any test concentration. For example, candidate inhibitors are typically tested initially at a concentration of 1 mM. Where the candidate inhibitor is sensitive to degradation by exopeptidases, the candidate inhibitor is tested in the presence of bestatin (20 μg/mL) to prevent breakdown of the inhibitor by isopeptidases present in muscle extracts. Candidate inhibitors for testing in these assays may be selected based on their structural similarity to known inhibitors, for example, the E3α inhibitors described herein. Novel molecules can also be obtained and tested for inhibitory activity. Compound or extract libraries can also be screened.

PURIFICATION OF E1, E2, AND E3 ENZYMES

Genes encoding E1 (yeast, wheat, and human) have been cloned, and therefore they can be expressed in *E. coli* and purified as described earlier (McGrath et al., 1991, EMBO J., 10:227; Hatfield et al., 1990, J. Biol Chem., 265:15813; Handley et al., 1991, Proc. Natl. Acad. Sci., 88:258). E1 can also be purified from rat muscle extracts as described earlier for rabbit reticulocyte extracts (Hershko et al., 1983, 258:8206).

E2-F1, involved in the degradation of non-N-end rule substrates, has been purified from reticulocytes (Blumenfeld, 1994, J. Biol. Chem., 269:9574). Therefore, it can also be purified from rat muscles by using the same procedure. Recently, genes of several E2 enzymes have been cloned, including recombinant human/rabbit $E2_{14k}$ (N-end rule substrates) and E2-F1 (non-N-end rule substrates). These enzymes can be expressed in *E. coli* and purified as described earlier (Wing et al., 1995, Biochem. J., 305:125; Nuber et al., 1996, J. Biol. Chem., 271:2795).

E3α, involved in the recognition of N-end rule substrates, can be purified using affinity chromatography on immobilized protein substrates (Reiss and Hershko, 1990, J. Biol. Chem., 265:3685).

MEASUREMENT OF E1 OR E2 ENZYME ACTIVITY

Thiol-ester assay. To study whether a typical inhibitor affects the activities of E1 or E2, the capacities of E1 or E2 to form thiol-ester bonds with ^{125}I-ubiquitin is measured (Blumenfeld, 1994, supra). E1-S-Ub or E2-S-Ub thiol-esters appear on SDS gels as ^{125}I-labeled bands of molecular weights corresponding to the mass of E1 plus the mass of ^{125}I-ubiquitin or to the mass of E2 plus the mass of ^{125}I-ubiquitin. These bands appear in the absence of reducing agent and disappear following the addition of a reducing agent (dithiothreitol or β-mercaptoethanol). In short, ubiquitin thiol-ester formation assays are carried out using either F-II, or purified E1 alone, or purified E1 and E2, in a buffer containing 50 mM Tris, pH 7.5, 5 mM $MgCl_2$, 2 mM ATP, ^{125}I-labeled ubiquitin (1 μg) and a potential inhibitor. Following the incubation at 37 °C for 5 minutes, the samples are divided into two portions. A sample buffer containing 0.5% SDS but not β-mercaptoethanol is added to one portion, and a sample buffer containing β-mercaptoethanol is added to the other. The β-mercaptoethanol containing samples are boiled for 5 minutes and SDS-PAGE analysis is then carried out at 4 °C at 5 mA, using a buffer in which the SDS concentration is reduced to 0.5%. Gels are dried and autoradiographed and the effect of added inhibitor is then checked by comparing the amount of thiol-esters formed in the absence or presence of a particular inhibitor.

MEASUREMENT OF E3 ENZYME ACTIVITY

E1 and E2 are rapidly inactivated by iodoacetamide treatment, while by contrast, E3 is much more resistant to this sulfhydryl blocking agent. See, Hershko et al. J. Biol. Chem. 258:8206-8214 (1983) hereby incorporated by reference in its entirety. Using iodoacetamide treatment, one can compare the activity of E3 in different rat muscle extracts as follows: To inactivate endogenous E1 and E2 enzymes, muscle extracts are treated with 5mM iodoacetamide for 5 minutes at 37°C (the conditions under which E1 and E2 lose most of their activities). Following incubation, dithiothreitol is added to the extracts at a concentration of 40 mM. The extracts are then dialyzed against the 50mM TRIS buffer at pH 7.6, 5mM $MgCl_2$ and 2 mM DTT (to remove iodoacetamide from extracts). To ensure that E1 and E2 enzymes are inactivated by the above treatment, the ubiquitin conjugation to ^{125}I-lysozyme in these dialyzed extracts and upon addition of either purified E1 or E2 alone can be checked. Purified E1 and $E2_{14k}$ are then added simultaneously to these treated extracts at fixed concentrations. Differences in E3 activity of these extracts are then compared by the ability of the extracts to form ubiquitin conjugates to ^{125}I-lysozyme.

MEASUREMENT OF PROTEIN DEGRADATION IN MUSCLE EXTRACTS

1. *Degradation of soluble endogenous proteins.* Reaction mixtures (100 µl) contain: 50 mM Tris pH 7.6, 5 mM $MgCl_2$, 2 mM DTT, 2 mM ATP, 10 µg of ubiquitin, and approximately 100 µg of protein of Fraction II. Following the incubation at 37 °C for 2 hours, the reaction is terminated by the addition of an equal volume of 20% TCA. The reaction mixtures are centrifuged for 3 minutes in an Eppendorf centrifuge, and the amount of tyrosine generated in the supernatant is measured fluorometrically by the method of Waalkes and Udenfriend. For inhibition studies, reaction mixtures also included a dipeptide or amino acid methyl ester inhibitor at a concentration of 1 mM and bestatin at a concentration of 20 µg/ml to prevent rapid destruction of the dipeptide inhibitor.

2. *Degradation of radiolabeled protein substrates.* Breakdown of exogenous ^{125}I-labeled protein is measured in the crude muscle extracts by following the release of TCA-soluble radioactivity using a gamma-counter. To specifically study protein degradation that is dependent upon N-end rule E2 and E3 enzyme activity, N-end rule pathway substrates, i.e. proteins with charged or bulky hydrophobic amino terminal residues, are employed in these experiments. As described herein, suitable substrates for these assays include lysozyme and lactalbumin.

MEASUREMENT OF PROTEIN DEGRADATION IN WHOLE CELLS

Once an inhibitor is identified which appears to be an effective inhibitor of ubiquitination in cell-free assays, it may be desirable to test that inhibitor in a cellular assay. Thus, cells are transformed with a genetic construct encoding a Ub—X—β-galactosidase fusion protein, wherein X is a "destabilizing" N-terminal residue according to the N-end rule. Cells are grown in culture in the presence or absence of a candidate inhibitor, harvested, and β-galactosidase enzyme activity in the cell extracts is determined according to reported procedures (Bachmair et al, 1986, Science, 234:179; Bartel, et al, 1990, 9:3179; Richter-Ruoff et al., 1992, FEBS Letters, 302:192; Grant et al. J. Immunology, 155:3750-58 (1995)).

MEASUREMENT OF PROTEIN DEGRADATION IN INCUBATED MUSCLES

To confirm the ability of inhibitors identified by these methods to reduce protein degradation in intact muscles, candidate inhibitors are then tested in vitro in incubated muscles from rats. In such experiments with rats, the soleus or extensor digitorum longus muscles from one leg can be incubated with an inhibitor, while the contralateral, identical muscle serves as a control. The advantage of such approaches is that they are highly sensitive, inexpensive, and do not require isotopic labeling of animals (Kettelhut et al, Diabetes/Metab. Rev., 1988, 4:751; Furuno et al., J. Biol. Chem., 1990, 265:8550). With experience, it is easy with six animals to demonstrate statistically significant changes in overall protein breakdown or synthesis as small as 10-15%. It can be calculated from the average turnover time of muscle proteins that even changes in this magnitude in proteolysis could be of therapeutic benefit; if maintained for 2 weeks, a 15% reduction in proteolysis by itself should lead to at least a doubling of mass of a denervated muscle. It is also of interest to follow the effects of the inhibitor on breakdown of myofibrillar proteins, which constitute 60% of the muscle mass and represent the major protein reserve in the organism. These proteins are lost differentially upon denervation, fasting or other catabolic conditions or diseases (Furuno et al., J. Biol. Chem. 1990, 265:8550). The degradation of myofibrillar components can be followed specifically by measuring 3-methylhistidine (3-MH) release from muscle proteins, which is a specific assay for breakdown of actin (Furuno et al, 1990, 265:8550); Lowell et al., Biochem. J., 1986, 234:237). It is of particular importance to carry out such studies with muscles undergoing denervation (disuse) atrophy or from fasted or endotoxin-treated (febrile) animals. In such tissues, overall protein breakdown is enhanced, and thus they closely mimic the human disease, but can be studied under well-defined in vitro conditions.

MEASUREMENT OF PROTEIN DEGRADATION IN AN ANIMAL

Inhibitors can also be tested for their ability to reduce muscle wasting in vivo. Urinary excretion of the modified amino acid 3-methylhistidine (3-MH) is a well-characterized method for studying myofibrillar protein degradation in vivo (Young and Munro, Federation Proc., 1978, 37:2291 hereby incorporated by reference in its entirety). 3-Methylhistidine is a post-translationally modified amino acid which cannot be reutilized for protein synthesis, and it is only known to occur in actin and myosin. It occurs in actin isolated from all sources, including cytoplasmic actin from many different cell types. It also occurs in the myosin heavy chain of fast-twitch (white, type II) muscle fibers, but it is absent from myosin of cardiac muscle and myosin of slow-twitch (red, type I) muscle fibers. Due to its presence in actin of tissues other than skeletal muscle, other tissues will contribute to urinary 3-MH. Skeletal muscle has been estimated to contribute 38-74% of the urinary 3-MH in normal rats and 79-86% of the urinary 3-MH in rats treated with corticosterone (100 mg/kg/day subcutaneously) for 2-4 days (Millward and Bates, 1983, Biochem. J., 214:607; Kayali et al., 1987, Am. J. Physiol., 252:E621).

Treatment with high doses of glucocorticoid or triiodothyronine (T3), or cecal ligation and puncture or implantation with tumor such as Yoshida Hepotoma can be used to induce a state of muscle wasting in rats. Treating rats with daily subcutaneous injections of corticosterone (100 mg/kg) causes an increase of approximately 2-fold in urinary 3-MH. The increase in excretion of 3-MH is transient, with a peak increase after 2-4 days of treatment and a return to basal values after 6-7 days of treatment (Odedra et al., 1983, 214:617; Kayali et al, 1987, 252:E621). Glucocorticoids have been shown to activate the ATP-ubiquitin-dependent proteolytic pathway in skeletal muscle (Wing and Goldberg, 1993, Am. J. Physiol., 264:E668). Ubiquitination inhibitors identified according to the invention described herein are thus expected to inhibit the muscle wasting that occurs after glucocorticoid or T3 treatment.

It is to be understood that the experimental protocol outlined above can be modified by those skilled in the art based upon the teachings herein to be adapted to include muscle tissue from different animal models including rat, rabbit, and mouse.

EXAMPLE I

In extracts of muscles of animals subject to high doses of thyroid hormones, e.g., hyperthyroidism, ubiquitin conjugation is faster than in untreated controls. In experimental models in which muscle breakdown is suppressed, for example, in thyroidectomized or hypophysectomized rats, ubiquitin conjugation was found to be slower than in untreated controls.

Briefly, extracts of muscle from hypophysectomized rats were prepared as described above using a cell-free system in which ubiquitin conjugation was monitored using exogenous labeled ubiquitin. Fig. 1 is a graph in which the rates of formation of ubiquitin-protein conjugates were measured at different times after exogenous ubiquitin was added to the extracts. "C" represents control extract, in which a normal level of ubiquitination occurs, and "H" represents hypophysectomized extract, in which an abnormally low level of ubiquitination occurs.

EXAMPLE II

The following example presents data demonstrating that arginine-methyl ester, as an inhibitor of the N-end rule ubiquitin conjugating enzyme E3α, will specifically suppress exogenous ubiquitination, whereas alanine-methyl ester will not.

Fig. 2 is a computer generated autoradiogram in which the amounts of labeled ubiquitin were measured in muscle extracts from muscles of control ("C") or hypophysectomized ("H") rats. In one set of C and H lanes, the dipeptide arginine-methyl ester (Arg ME, 1mM) is shown to reduce ubiquitin conjugation to soluble proteins in the control sample to approximately the level of ubiquitin conjugation that occurs in the hypophysectomized rat muscle extract. In another set of C and H lanes, the dipeptide analog alanine-methyl ester (Ala ME, 1mM) is shown to have no effect on ubiquitin conjugation.

Therefore, the results presented in Fig. 2 demonstrate that ubiquitination and muscle proteolysis can be specifically suppressed with arginine-methyl ester (or leucine-methyl ester, not shown) but not with alanine-methyl ester. Similarly, the dipeptide leucine-alanine, which binds to E3α, suppresses ubiquitination, but the isomer alanine-leucine, which does not bind to E3α, has no effect on ubiquitination.

EXAMPLE III

Ubiquitin-conjugation is suppressed by thyroidectomy and hypophysectomy. The decrease in muscle proteolysis observed in thyroidectomized or hypophysectomized rats can be observed by treatment of the animals with thyroid hormones, triiodothyronine (T_3) or thyroxine (T_4). In fact high levels of these hormones cause excessive protein breakdown and muscle wasting as seen in hyperthyroid patents. Muscle extracts from control ("C"), thyroidectomized ("TX"), T3-treated thyroidectomized ("T3"), hypophysectomized ("H") and T_3 treated hypophysectomized ("T_3") rats were prepared and the rate of ubiquitination to soluble proteins was measured. Thyroidectomized rats were treated with T_3 (20μg/100g body wt.) for 7 days and the hypophysectomized rats (100μg

T_3/100 g body wt.) for 4 days prior to killing. The control rats were administered an equal volume of physiological saline. Figs. 3 and 4 are computer generated autoradiograms showing the results wherein labeled exogenously added ubiquitin is measured over time. Treatment with T_3 not only restored the normal growth rate and body temperature in the thyroidectomized animals (from 35.7°C to 36.7°C, $p<0.01$), but also stimulated Ub conjugation to muscle proteins back toward levels seen in controls.

The results demonstrate that ubiquitin-conjugation is suppressed by thyroidectomy, as it is by hypophysectomy, and that treatment of the rats for 7 days with high doses of triiodothyronine (T3), which enhances muscle proteolysis to normal levels, stimulates ubiquitin conjugation.

EXAMPLE IV

Inhibitors of E3α also suppress protein ubiquitination induced by thyroid hormones in normal and T3-treated rats. Muscle extracts from control ("C"), thyroidectomized ("TX) and T3-treated thyroidectomized ("T3") rats were prepared and assayed using electrophoresis techniques known in that art and described earlier for the level of ubiquitination to soluble proteins in the presence or absence of arginine-methyl ester (1mM) or alanine-methyl ester (1mM). Fig. 5 is a bar graph showing the results of this experiment. It was found that the E3α inhibitor arginine-methyl ester suppressed ubiquitination in both normal and T3-treated rats to the basal level of ubiquitination of muscle extracts from thyroidectomized rats. The inactive homolog, alanine-methyl ester had no effect on the level of ubiquitination in any of the extract preparations.

EXAMPLE V

In extracts of muscles from rats afflicted with sepsis ("S") induced by cecal ligation and puncture to cause peritonitis and bacteriemia, rates of ubiquitination of endogenous muscle proteins were found to be enhanced relative to rates of ubiquitination in control ("C") rats. Fig. 6 is a computer generated autoradiogram showing the results of measuring labeled conjugation of exogenous ubiquitin to muscle proteins over time.

EXAMPLE VI

Muscle extracts from control ("C") and septic ("S") rats were prepared and assayed for levels of ubiquitination in the presence or absence of known inhibitors of E3α.

Fig. 7 is a computer generated autoradiogram in which labeled ubiquitin was measured in extracts from control and septic rat muscle extracts. The results show that both arginine-methyl

ester (1mM) and leucine-methyl ester (1mM) reduce the increased ubiquitin conjugation found in extracts of atrophying muscles from septic animals to approximately the levels of ubiquitin conjugation found in control muscles. The homolog alanine-methyl ester was found to have no effect on the level of ubiquitination.

EXAMPLE VII

As reported by Fagan et al., Biochem J., 243: 335-343 (1987), the majority of proteins in soluble extracts (Fraction II) of rabbit skeletal muscles are subject to degradation via the ATP-dependent ubiquitin proteasome pathway. Soluble Fraction-II (5mg/ml) from skeletal muscles of normal rabbits were incubated at 37 °C in the absence or presence of 2 mM ATP and/or ubiquitin (200 μg/ml). Samples were withdrawn, TCA added and the amount of tyrosine released due to breakdown of soluble proteins was measured. As can be seen in Fig. 8, the addition of ATP and ubiquitin to the dialysed Fraction-II resulted in the increased formation of free tyrosine molecules indicating that ATP and ubiquitin stimulate the degradation of endogenous proteins completely to amino acids. This process occurred at a linear rate for 2 hours at 37°C and was stimulated 3- to 6- fold by ATP.

EXAMPLE VIII

As demonstrated below, inhibitors of components of the N-end rule pathway for protein ubiquitination reduce the ATP-Ub-dependent degradation of soluble proteins in rabbit muscle extracts. Soluble Fraction-II (5 mg/ml) from rabbit skeletal muscles were incubated at 37°C for 2 hours in the presence of 2 mM ATP and 200 μg/ml ubiquitin. Dipeptides or methylesters at 2 nm concentration were added to various reaction mixtures as indicated in Table 1 below. Bestatin (20μg/ml) was also added to all reaction mixtures in order to protect added dipeptides from endogenous aminopeptidases. 100% proteolytic activity was ascribed for the value measured in a system to which inhibitors were not added. 700 pMoles of tyrosine were released under this condition.

Table 1

	Inhibitor	% Inhibition of Degradation
1.	None	0
2.	Leucine methyl ester	40
3.	Arginine methyl ester	70
4.	Alanine methyl ester	15
5.	Phenylalanine-Alanine	40
6.	Alanine-Phenylalanine	10

As shown in Table 1, addition of 1 mM arginine methyl ester, an inhibitor of the N-end rule ubiquitin-conjugating enzyme E3α, to Fraction-II inhibited up to 70% of the total ATP-ubiquitin-mediated degradation of endogenous proteins, as measured by the release of free tyrosine molecules. The ester or dipeptide derivatives of hydrophobic amino acids such as leucine methyl ester or phenylalanine-alanine also inhibited up to 40% of the ATP-stimulated degradation of endogenous proteins while alanine methyl ester or alanine-phenylalanine at the same concentration caused little inhibition.

Further studies were performed according to the following experimental protocols.

Muscle extract preparation. Male New Zealand white rabbits (3-4 kg) were killed by lethal injection of sodium pentabarbitol, and the extracts from psoas muscles were prepared. Homogenates were centrifuged at 30,000xg for 30 minutes to remove myofibrils. "Crude extracts" were prepared by centrifuging the supernatants at 100,000xg for I hour and were either studied directly or fractionated on DEAE-cellulose into Fraction-II (F-II), the resin-bound material, which contained the proteasomes and most of the enzymes required for Ub -conjugation, and Fraction-I (F-I), the flow-through which contained Ub and 70% of cell proteins. Both crude extracts and Fraction-II were then dialyzed against buffer containing 20 mM Tris (pH 7.6), 2 MM DTT, 5 mM $MgCl_2$ and 10% glycerol and stored at -70°C until use.

Protein degradation assays in skeletal muscle extracts. Degradation of endogenous proteins in crude extracts and Fraction-II was measured by assaying the free tyrosine in the TCA-soluble supernatant. Reaction mixtures contained in a volume of 100μl: 20 mM Tris pH 7.6, 5 mM MgCI2, 2 mM DTT, ATP-regenerating system (10μg creatine phosphokinase and 10 mM creatine phosphate), 1 mM ATP, 25μg of Ub and approximately l mg of dialyzed crude extract or Fraction II. Following incubation at 37°C for 2 hours, the reactions were terminated by the addition of an equal volume of 20% TCA. The reaction mixtures were centrifuged and the amount of tyrosine

generated in the supernatant was measured by fluorescence spectroscopy. When degradation of ^{125}I-actin or ^{125}I-lysozyme in the crude extracts was studied, the reaction mixture contained 3 μg of labeled proteins and their degradation was measured by following the release of TCA-soluble radioactivity using a gamma counter.

Measurements of ^{125}I-Ub-conjugation to muscle proteins. For ubiquitination assays, the Fraction-II were further centrifuged for 6-8 hrs at 100,000g to deplete most of the proteasomes. The preparation (60μg in a volume of 25μl) was then incubated with ^{125}I-Ub at 37°C in a buffer containing 20 mM Tris pH 7.4, 1 mM DTT, 5 mM MgCI2 and 2 mM ATP$_\gamma$S in the absence or presence of various inhibitors. Following incubation at 37°C at desired time periods, the reactions were terminated by addition of sample buffer and SDS-PAGE was performed. The gels were then dried and auto-radiographed.

To further confirm that the N-end rule pathway contributed to degradation of endogenous proteins, various competitive inhibitors of E3α were added to the rabbit muscle extracts as prepared above and the average data presented in Table 2 below. As indicated, the reaction mixtures included L-amino acid dipeptide or methylester inhibitors of E3α at 2mM. To prevent hydrolysis of the added dipeptide, 20 µg/ml bestatin, an inhibitor of aminopeptidases, was added to all reaction mixtures, including controls. bestatin alone reduced tyrosine production slightly (less than 20%). 100% proteolytic activity is the value measured in the absence of any inhibitors. Typically, crude extracts produced 150 pmoles of tyrosine in the absence of ATP, 550 pmoles in its presence, and for Fraction-II, 120 pmoles of tyrosine was generated in the absence of ATP, and 700 pmoles in the presence of ATP and Ub after 2 hours. The ATP-independent proteolysis was subtracted from all samples, and the results were normalized to 100%. Since the addition of inhibitors had no effect on degradation in the absence of ATP and/or ubiquitin, the data are not shown. The data shown in Table 2 were obtained in a single experiment and are the averages of triplicate determinations which agreed within 10%. All experiments were repeated at least three times with similar results.

Table 2

	Additions	% Inhibition of Degradation	
		Crude	Fraction II
1.	None	0	0
2.	Arginine methyl ester	50	45
3.	Leucine methyl ester	30	25
4.	Alanine methyl ester	10	5
5.	Phe-Ala	30	25
6.	Ala-Phe	5	5

As indicated in Table 2, addition of arginine methyl ester (2 mM), reduced the ATP-dependent degradation of soluble muscle proteins by 50%. The inhibitor of the hydrophobic site on E3α, leucine methyl ester, also reduced proteolysis but to a lesser extent (30%). In contrast, alanine methyl ester, which does not inhibit E3α, had little or no effect on the breakdown of muscle proteins. Similarly, the dipeptide inhibitor of E3α, Phe-Ala, also reduced ATP-stimulated degradation of endogenous proteins by 30%, but its isomer, Ala-Phe, had very little effect on the degradation. There was also a low amount of ATP independent proteolysis in these preparations, but it was not affected by the dipeptides or amino acid esters (data not shown).

A very similar inhibition of proteolysis was seen after these cell-extracts were fractionated by DEAE chromatography. Most cell proteins (Fraction-I) including Ub flow through the column but about 30% of cell proteins was bound and eluted with high salt. This material (Fraction-II) contained El, $E2_{14k}$, E3α and proteasomes. When ATP and Ub were added to Fraction-II, they stimulated the breakdown of endogenous proteins 3- to 5-fold and as shown in Table 2, this ATP-Ub-stimulated degradation was inhibited when arginine methyl ester, Lys-Ala or Phe-Ala was added, while alanine methyl ester, Ala-Lys or Ala-Phe which do not inhibit E3α, were ineffective. In fact as shown in Table 2, the pattern of inhibition in Fraction-II with the dipeptide and methyl ester inhibitors of E3α was almost identical to that seen in crude extracts.

As shown in Table 3 below, the dipeptide and amino acid ester with basic N-termini which reduced breakdown of endogenous muscle proteins inhibited similarly the rapid degradation of ^{125}I-lysozyme, a model substrate of the "N-end" system which contains a lysine at its N-terminus. Crude extracts were preincubated for 5 minutes at 37°C with the various inhibitors. ^{125}I-labeled substrates, 1mMATP and the ATP-regenerating system was then added, and the incubation was continued further for 2 hours. Additional conditions were similar to those outlined with reference

to the data presented in Table 2. Degradation of the ^{125}I-labeled substrates was then measured and compared to that in extracts without inhibitors. The degradation of the labeled proteins was very low in the absence of ATP. Since the various inhibitors had negligible effect on this ATP-independent degradation, these data are not shown.

In the crude muscle extract, Lys-Ala and arginine methyl ester selectively inhibited the ATP-dependent degradation of ^{125}I-lysozyme while Ala-Lys, Leu-Ala or alanine methyl ester had no effect on ^{125}I-lysozyme breakdown. Ub-mediated degradation of proteins with N-α-acetylated termini involves a different E3 and is not sensitive to inhibitors of E3α. To confirm the specificity of these inhibitors, their effects on the breakdown of exogenously added ^{125}I-actin were studied, which is also degraded in muscle extracts by the Ub-proteasome pathway, but unlike lysozyme, have an N-α-acetylated termini. At concentrations which inhibited lysozyme degradation by 60%, neither arginine methyl ester nor Lys-Ala reduced the degradation of ^{125}I-actin as shown in Table 3 or of added ^{125}I-myosin (data not shown). Without ATP, 1% of ^{125}I-actin and 0.5% of ^{125}I-lysozyme were degraded, and with ATP, 4% of ^{125}I-actin and 4% of ^{125}I-lysozyme were degraded.

Table 3

	Additions	% Inhibition of Degradation	
		^{125}I-Lysozyme	^{125}I-Actin
1.	None	0	0
2.	Arginine methyl ester	60	15
3.	Leucine methyl ester	15	5
4.	Alanine methyl ester	10	10
5.	Lys-Ala	50	10
6.	Phe-Ala	10	12
7.	Ala-Phe	10	5

EXAMPLE IX

In order to determine the effect of inhibitors on the ubiquitination of endogenous proteins in muscles of control and cachetic rats, test rats were implanted with Yoshida Ascites Hepatoma AH 130 (YAH) as described in Baracos et al. Am. J. Physiol. 1995; E996-E1006. Specifically, female Sprague Dawley rats of the Buffalo strain were implanted with 100 µl of ascites fluid containing YAH cells from a single donor animal. Control rats were implanted with equal volume of buffer. On days 3 and 5 after tumor implantation, rats were killed by CO_2 asphyxiation and leg muscles (soleus, quarter-diaphragm, atria and extensor digitorum longus all together) were

dissected for use in an ubiquitin conjugation assay previously described. Fig. 10 shows that in muscle extracts (Fraction-II) from cachetic rats implanted with YAH, rates of ubiquitination of endogenous muscle proteins are increased relative to rates of ubiquitination in control rats. Fig. 10 is an autoradiogram of a typical SDS gel showing the results of measuring ubiquitin conjugation over time in control and YAH bearing rat muscle extracts.

Similar studies were also performed to determine ^{125}I-Ubiquitin conjugation to endogenous soluble proteins as well as to exogenously added ^{125}I-lysozyme in muscle extracts from control mice, mice bearing colon-26 tumor and mice after tumor removal as distinguished from the experiments conducted in the rat models. The results in Figs. 11 and 12 demonstrate increases in ubiquitin conjugation in the tumor bearing mice as compared to the controls. Moreover, the rate of ubiquitin conjugation fell to normal control levels when the tumor was removed and the animals regained normal weight.

EXAMPLE X

The rates of ubiquitination were then measured in extracts from control and cachetic rats (3 days and 5 days after YAH implantation) in the presence or absence of known inhibitors of E3α. The results presented in Fig. 13 show that arginine methyl ester (1mM) reduced the increased ubiquitin conjugation found in Fraction-II extracts of skeletal muscles from the YAH bearing rats to approximately the levels of ubiquitin conjugation found in the control rats. The homolog alanine methyl ester, which does not bind E3α, was ineffective in reducing the increased ubiquitin conjugation.

EXAMPLE XI

A number of protein substrates subject to degradation via the N-end rule pathway, specifically those with acidic N-termini, undergo modification by the addition of arginine or leucine to their N-terminal residue that leads to their binding by E3α for ubiquitin conjugation. This reaction requires arginine-tRNA protein transferase as the arginine form or leucyl-tRNA protein transferase as the leucine form (collectively "tRNA"). Addition of RNase A to the reaction mixture inhibits protein modification and, therefore, the ubiquitin-proteasome mediated degradation by destroying the tRNA. Ferber et al., J. Biol. Chem. 261:3128-3134 (1986); Ferber et al. Nature 326: 808-811 (1987).

To demonstrate that tRNA is necessary for ATP-stimulated degradation of endogenous proteins in rabbit muscle extracts, reaction mixtures were prepared containing a volume of 100

µl:50mM Tris pH 7.6, 5mM $MgCl_2$, 2mM DTT, 2mM ATP, 10 µg of ubiquitin and approximately 100 µg of rabbit muscle protein either as a crude extract or as Fraction-II. Following incubation at 37°C for 2 hours, the reaction was terminated by the addition of an equal volume of 20% TCA. The reaction mixtures were centrifuged for 3 minutes in an Eppendorf centrifuge and the amount of tyrosine generated in the supernatant was measured. For RNase A inhibition assay, the reaction mixtures were preincubated for 30 minutes with 0.02 U of RNase A prior to addition of ATP and ubiquitin. In this reaction, the extracts were preincubated with RNase A (0.02U) and then the inhibitor (0.05U) was added to inhibit the nuclease. Subsequently, tRNA (-) was added. 100% proteolytic activity is the value that was measured in a system to which inhibitors were not added. 700 pMoles of tyrosine were released under this condition. The percent inhibition of degradation is presented in Table 4 below.

Table 4

	Additions	% Inhibition of Degradation	
		Crude	Fraction-II
1.	None	0	0
2.	RNase	35	50
3.	RNase inactivated with RNase Inhibitor	5	5
4.	RNase then RNase Inhibitor then tRNA	30	25
5.	DNase-I	5	0

A separate set of experiments using the crude extract were conducted with the following average data presented in Table 5 below. The reaction mixtures were preincubated for 30 minutes with the 0.02U of RNase A (from Bovine pancreas) prior to the addition of ATP and Ub. Crude extract was also preincubated with RNase A (0.02U) and then excess human placental Rnase inhibitor (0.05U) was added to inactivate the nuclease. Subsequently, tRNA from fetal calf liver (0.13U) was added. The concentrations of bovine pancreas DNase I was 5U.

Table 5

	Additions (2 mM)	% Inhibition of Degradation	
		Crude	Fraction-II
1.	None	0	0
2.	RNase	25	--
3.	RNase inactivated with RNase Inhibitor	5	--
4.	RNase then RNase Inhibitor then tRNA	15	--
5.	DNase-I	0	--

Treatment of the rabbit muscle extracts with RNase A prior to the addition of ubiquitin and ATP markedly inhibited the ATP-ubiquitin stimulated degradation of endogenous proteins. Several observations further supported that the inhibitory effects of the RNase A was due to its enzymatic activity: 1) DNase I at high concentrations did not inhibit the degradation of endogenous proteins; 2) when the activity of RNase A was inhibited by preincubating the RNase A with human placental ribonuclease inhibitor before adding the enzyme to the extract, the inhibitory effect of RNase A was completely abolished; and 3) when muscle extracts were treated with RNase A, the inhibitor added to inactivate the nuclease, and tRNA subsequently added, the tRNA was found to restore proteolysis. However, when ribonuclease was not inhibited before addition of the tRNA, no restoration was observed. Therefore, tRNA is necessary for ATP-dependent degradation of proteins in rabbit muscle extract and RNase A inhibited ATP-Ub-dependent degradation of endogenous proteins in muscle extract (Fraction-II) by inhibiting ubiquitin conjugation to muscle protein substrates.

EXAMPLE XII

Studies using ^{125}I-ubiquitin also demonstrate that RNase A inhibits ubiquitin conjugation to rabbit and rat muscle extract proteins. Proteasome depleted Fraction-II (50μg) in a volume of 25μl were incubated with ^{125}I-ubiquitin (125μg/ml) at 37°C in a buffer containing 10mM Tris pH 7.4, 1 mM DTT, 5mM $MgCl_2$ and 2 mM ATPγS. After 60 minutes of incubation, the samples were withdrawn, and the amount of conjugates formed were analyzed using 12% SDS-PAGE. For RNase A inhibition assay, the reaction mixtures were preincubated for 30 minutes with 0.01 U of RNase A prior to addition of ATPγS and ubiquitin. In some reactions, the RNase A activity was inhibited by pretreatment with RNase A inhibitor (Human placental ribonuclease inhibitor, 0.03U). The concentration of DNase used was 5U.

Fraction-II was used in order to remove endogenous ubiquitin. Also to avoid the unwanted degradation of conjugates by the 26S proteasome complex and also to remove the isopeptidase(s) associated with the 26S proteasome complex, extracts of proteasomes were depleted by ultracentrifugation. In addition, ATP was replaced with ATPγS, since this nucleotide supports Ub-conjugation, but not proteolysis by the 26S proteasome. Incubation of ^{125}I-Ub with rabbit or rat muscle Fraction-II resulted in the formation of ^{125}I-Ub-protein conjugates as detected by the appearance of new high molecular weight ^{125}I-labeled bands (<8kDa) in SDS gels, which appeared only in the presence of ATPγS. The formation of Ub-protein conjugates was linear for up to 60 minutes at 37°C.

As shown in Table 6 below, addition of RNase A, but not DNase-I to rabbit or rat muscle Fraction-II inhibited markedly ^{125}I-ubiquitin conjugation to soluble proteins in the extracts. However, when the enzymatic activity of RNase A was inhibited with human placental ribonuclease inhibitor, before adding them to the extract, no reduction in ^{125}I-ubiquitin conjugation was observed suggesting that the N-termini of proteins in muscle extracts are modified by arginylation, prior to recognition by the N-end rule ubiquitin-conjugating enzyme, E3α.

Table 6

	Additions	% Inhibition of Ubiquitination	
		Rabbit	Rat
1.	None	0	0
2.	RNase A	60	55
3.	RNase A inactivated with RNase A inhibitor	15	13
4.	DNase A	5	0

Additional experiments were performed showing percent inhibition of ubiquitination with RNase A, RNase A pretreated with RNase inhibitor and DNase I, as well as, with inhibitors. That data is presented in Table 7 below:

Table 7

	Additions	% Inhibition of Ubiquitination	
		Rabbit	Rat
1.	None	0	0
2.	Arginine methyl ester	40	45
3.	Leucine methyl ester	30	25
4.	Alanine methyl ester	5	0
5.	Phe-Ala	25	30
6.	Ala-Phe	10	10
7.	RNase A	30	25
8.	RNase A inactivated with RNase A inhibitor	5	7
9.	DNase A	5	0

Values shown above are relative rates of ubiquitination of endogenous proteins in the absence or presence of various inhibitors of E3α. 100% is the amount of ^{125}I-radioactivity incorporated, in the absence of any inhibitor, into higher molecular weight forms (defined arbitrarily as ^{125}I-Ub migration with molecular weight greater than 20 kDa). This data shows significant percent inhibition by arginine methyl ester and leucine methyl ester and Phe-Ala. In contrast, alanine methyl ester or Ala-Phe, which do not inhibit E3α, did not significantly affect the ubiquitination of muscle proteins.

EXAMPLE XIII

Additional studies were performed to demonstrate that inhibitors of N-end rule ubiquitin conjugating enzymes were effective in reducing ubiquitination of rat muscle proteins as shown in Fig. 14. In the extracts (Fraction-II) from both control and septic rats (A), addition of the inhibitor of E3α, arginine methyl ester, reduced ^{125}I-Ub conjugation to soluble proteins. However, ubiquitination was inhibited to a much greater extent in extracts from septic animals than in those from control animals. As a result most of differences in rates of Ub conjugation between the two groups were eliminated by arginine methyl ester. Similarly, leucine methyl ester, which binds to a distinct site on E3α and also reduces proteolysis in normal rabbit muscles suppressed the high rate of Ub conjugation in the extracts from septic animals much more than in controls, and thus reduced the differences in the ubiquitination between these preparations. In contrast, alanine methyl ester, which does not inhibit the "N-end" pathway, had no effect on the ubiquitination of proteins in either extract.

Similar experiments were carried out with extracts from hepatoma-bearing and control rats. The addition of arginine methyl ester (B) or the dipeptide Lys-Ala (data not shown) suppressed most of the increased ubiquitination in muscle extracts of tumor-bearing rats, but had much less inhibitory effect in control extracts. As a result, these agents eliminated most of the differences in

Ub conjugation between control and atrophying muscle extracts. In contrast, alanine methyl ester or Ala-Lys had no effect on the protein ubiquitination in either groups.

Because these findings indicated that the enhanced Ub-conjugation in the atrophying muscles is largely through the N-end rule pathway for protein degradation, an experiment was performed to determine whether the suppression of Ub conjugation that accompanies the fall in muscle proteolysis in hypothyroid (C) and thyroidectomized (D) animals, effect activity of the N-end rule pathway. Addition of arginine methyl ester to the extracts of control rat muscles, caused a 40-50% inhibition of Ub-conjugation, but only a 10-20% inhibition of the low amount of ubiquitination in the extracts from thyroidectomized or hypophysectomized rats. As a consequence, with this inhibitor present, the rates of Ub-conjugation were similar in these different extracts. In contrast, alanine methyl ester had no effect on rates of ubiquitination. Thus, the fall in Ub- conjugation in hypothyroid animals seems to be mainly due to a suppression of the N-end rule pathway. Furthermore, T_3-treatment of the thyroidectomized or hypophysectomized rats not only restored rates of Ub-conjugation as shown previously, but also enhanced the sensitivity of this process to the inhibitors of E3α (C and D).

EXAMPLE XIV

Differences in the ubiquitination of cell protein arise either through activation (or inhibition) of enzymes comprising the N-end rule pathway or through changes in the protein substrates in the cell, for example by modifications of muscle proteins that make them better substrates for ubiquitin conjugating enzymes such as E3α. To test whether the activity of these ubiquitination enzymes is enhanced during sepsis or in tumor-bearing rats, ubiquitination of ^{125}I-lysozyme, a model substrate of the N-end rule pathway, was measured in various extracts. As shown in autoradiograms of Figs. 15-18, Ub conjunction to this exogenous substrate which contains a lysine at its N-terminus, was about 2-fold higher in extracts from septic animals than in controls (Fig. 15) and 1.5-2 fold higher

in rats bearing the Yoshida Hepatoma for 3 to 5 days (Fig. 16). Furthermore, Ub conjugation to ^{125}I-lysozyme was reduced by about 50% below control rates in rat muscles following the thyroidectomy (Fig. 17) or hypophysectomy (Fig. 18), and the ubiquitination of ^{125}I-lysozyme in these extracts was restored back to levels seen in normal controls when these hypothyroid rats were treated with T_3.

EXAMPLE XV

Levels of ^{125}I-ubiquitin conjugation were determined in soluble muscle proteins of rats exhibiting various pathologic states including diabetes induced by treatment with streptozocin, YAH tumor presence, and chronic renal failure. As shown in the autoradiograms of Fig. 19, increased ubiquitin conjugation was observed in the soluble muscle proteins from the rats exhibiting the various pathologic states as compared to control animals, (col. 1 lane 1 = control "C", col. 1 lane 2 = diabetes "D"; col. 2 lane 1 = control, col. 2 lane 2 = YAH tumor bearing "YAH"; col. 3 lane 3 = control "C", col. 3 lane 2 = chronic renal failure "CRF") whereas ubiquitin conjugation was lowered below control levels in hypophysectomized animals (col. 4 lane 1 = control "C", col. 4 lane 2 = hypophysectomy "H").

Levels of the N-end rule ubiquitin conjugating enzyme $E2_{14k}$ was monitored in various disease states in rat muscle extract where protein degradation and ubiquitin conjugation are accelerated. As shown in Fig. 20, both amount and activity of E1 and $E2_{14k}$ as assayed by western blotting "E1W" and "E2W" showing band intensity and thiol-ester formation with ^{125}I-Ubiquitin increased in muscle from rats rendered diabetic by streptozotocin-induced diabetes. As shown in Fig. 21, both amount and activity of $E2_{14k}$ as assayed by western blotting and thiol-ester formation increased in muscle from rats with chronic renal failure exhibiting acidosis or uremia. Also, as protein degradation increased in individual muscles from rats with chronic renal failure as compared to control rats, levels of $E2_{14k}$ also increased. As shown in Fig. 22, the amount of $E2_{14k}$ as assayed

by western blotting increased in muscle from rats bearing Yoshida Ascites Heptoma as compared to control rats. As shown in Fig. 23, as levels of muscle protein degradation and ubiquitin conjugation decreased in hypophysectomized rats, both amount and activity of $E2_{14k}$ also decreased as measured by western blotting and thiolester formation.

Studies were also performed which confirmed that inhibitors of the N-end rule ubiquitin conjugating enzyme E3α abolished differences in ubiquitin conjugation between control rats and rats with chronic renal failure.

EXAMPLE XVI

Experiments were conducted to determine the effect on protein degradation in soluble extracts of rabbit muscle of increased levels of N-end rule ubiquitin conjugating enzymes, such as $E2_{14k}$ and E3. Each of purified E1, $E2_{14k}$ and E3α were added to rabbit muscle extracts in low amount of 2.5μg/2.5mg extract and a high amount of 10μg/2.5mg extract and the total amount of protein degradation was monitored by measuring the amount of tyrosine in pmoles released after 2 hours. A separate similar experiment was conducted in which the amount of E3α dependent proteolysis was monitored. The data is presented in Table 8 below.

Table 8

	Total Proteolysis		E3α-dependent	
Additions	Low	High	Low	High
None	200	200	110	110
E1	280	320	170	120
$E2_{14k}$	310	900	180	400
E3α	390	1280	260	820

As the data in Table 8 indicates, when the levels of the N-end rule ubiquitin conjugating enzymes $E2_{14k}$ and E3α were increased, the degradation of endogenous proteins increased. Further experiments were also conducted in which 2mM of a Lys-Ala and Phe-Ala combination inhibited proteolysis.

The invention provides novel cell-free systems for the screening and identification of inhibitors of muscle wasting which act by interfering with the function of components of the N-end rule pathway for ubiquitination. With the identification of such inhibitors, it is possible to therapeutically treat muscle wasting by improving nitrogen balance in various disease states.

The invention further provides for inhibiting the enhanced ubiquitination and thus excessive protein degradation that occurs in patients with cachexia. While mobilization of amino acids from muscle protein might appear beneficial in acutely ill patients, continued rapid muscle catabolism is clearly debilitating. Besides providing an adequate diet, there is no effective way of improving nitrogen balance in catabolic patients. One approach would be to partially inhibit the ubiquitin-proteasome pathway. Complete inhibition would be incompatible with life, but for most severely cachectic patients, partial suppression of muscle protein breakdown is likely to be beneficial by sparing essential cell proteins. Selective inhibition of the N-end rule ubiquitin conjugation pathway provides a therapeutic approach for the treatment of muscle wasting.

DETERMINATION OF EFFECTIVE INHIBITION

Embodiments of the present invention are designed to identify a candidate inhibitor of muscle wasting, i.e., a molecule that inhibits muscle protein degradation by the ubiquitin-proteasome pathway, by interfering with one of the components involved in the transfer of ubiquitin to a target molecule or the ligation of ubiquitin to the target molecule or the modification of the target molecule prior to recognition of the protein by an N-end rule ubiquitin conjugating enzyme.

To be an effective inhibitor according to the invention, a candidate inhibitor is one which will exhibit some significant degree of inhibition by the assays described herein. For example, a degree of greater than 15% inhibition of muscle protein breakdown by a candidate inhibitor in any one of the assays described herein will be considered an adequate level of inhibition by a candidate inhibitor to be considered useful according to the invention, although higher degrees of inhibition are preferred (e.g., in the range of 20%-50%, 40%-70% or even higher, e.g., in the range of 51%-100%).

It is expected that a candidate inhibitor that inhibits ubiquitination as measured by any of the methods described herein is useful if it inhibits at a level of greater than or equal to 60% relative to a control in a sepsis animal model, or a level of greater than or equal to 50% in a hyperthyroid animal model.

A candidate inhibitor that is truly effective as an inhibitor of muscle wasting in a disease in which muscle wasting is accelerated is one which when administered directly in a mammal afflicted with a disease that causes muscle wasting, reduces muscle wasting to any degree which ameliorates the condition to a medically noticeable degree. That is, an inhibitor need not cure the loss of muscle tissue completely by bringing the level of muscle wasting to a normal or near-normal level, although this result is desirable, but the inhibitor may reduce muscle wasting to any detectable degree to be considered useful. The effectiveness of an inhibitor will vary with the disease, delivery of the inhibitor, as well as the dosage. The delivery and dosage will be determined by the physician according to the disease state.

Accordingly one embodiment of the present invention includes a therapeutic composition for use in the treatment of diseases characterized by accelerated muscle wasting in which an inhibitor capable of interfering with the function of a component of the N-end rule pathway for protein ubiquitination so as to suppress ubiquitination of muscle protein is combined with a pharmaceutically acceptable carrier. In a preferred embodiment, the inhibitor is a dipeptide, isolated

or otherwise, having an amino terminal amino acid which mimics an abnormal amino terminus of a protein, and is substantially free of contaminants. The component of the N-end rule pathway for protein ubiquitination is preferably an N-end-rule ubiquitin conjugating enzyme such as an E2 or E3 enzyme or more particularly, $E2_{14k}$ or E3α. In an alternate embodiment, the component of the N-end rule pathway for protein ubiquitination is an N-terminal protein modifying enzyme such as arginyl-tRNA protein transferase or leucyl-tRNA protein transferase. Pharmaceutically acceptable/biologically compatible carriers may be, for example, in the form of a cream, ointment, lotion or spray for topical use, or in the form of a pill, tablet, capsule, or physiological solution, such as a salt solution, for internal administration.

For therapeutic uses, inhibitors or compositions thereof identified as described herein may be administered by any number of known routes, including orally, intravenously, intramuscularly, subcutaneously, topically, and by infusion. The amount of inhibitor administered will be determined according to the degree of muscle wasting exhibited in a diseased state, the physical nature of the recipient and whether such muscle wasting is systemic or localized, and will typically be in the range of about 1 μg/kg to 100 mg/kg body weight. A single dose of inhibitor or multiple doses, daily, weekly, or intermittently, is contemplated according to the invention.

OTHER EMBODIMENTS

Other embodiments will be evident to those of skill in the art. It should be understood that the foregoing detailed description is provided for clarity only and is merely exemplary. The spirit and scope of the present invention are not limited to the above examples, but are encompassed by the following claims.

What is claimed is:

1. A method of inhibiting muscle wasting in a mammal resulting from degradation of muscle protein via the N-end rule pathway, comprising interfering with function of a component of the N-end rule pathway for protein ubiquitination so as to suppress ubiquitination of the muscle protein.

2. A method of inhibiting degradation in cultured muscle cells of muscle protein targeted for degradation via the N-end rule pathway, comprising providing a muscle cell culture; and

contacting the culture with an agent that interferes with function of a component of the N-end rule pathway for protein ubiquitination so as to suppress ubiquitination of the muscle protein.

3. A method of inhibiting degradation in muscle cell extracts of muscle protein targeted for degradation via the N-end rule pathway, comprising providing a muscle cell extract; and

contacting the extract with an agent that interferes with function of a component of the N-end rule pathway for protein ubiquitination so as to suppress ubiquitination of the muscle protein.

4. The method of claim 1, 2, or 3 wherein the component of the N-end rule pathway for protein ubiquitination comprises an N-end rule ubiquitin conjugating enzyme.

5. The method of claim 4, wherein the N-end rule ubiquitin conjugating enzyme is an E2 enzyme.

6. The method of claim 5 wherein the E2 enzyme is $E2_{14K}$.

7. The method of claim 4 wherein the N-end rule ubiquitin conjugating enzyme comprises an E3 enzyme.

8. The method of claim 7 wherein the E3 enzyme is E3α.

9. The method of claim 1, 2, or 3 wherein the component of the N-end rule pathway for protein ubiquitination comprises an N-terminal protein modifying enzyme.

10. The method of claim 9 wherein the N-terminal protein modifying enzyme is arginyl-tRNA protein transferase or leucyl-tRNA protein transferase.

11. The method of claim 1, 2, or 3, wherein the muscle protein is from an animal afflicted with a muscle wasting condition selected from the group consisting of hyperthyroidism, AIDS- or cancer-related cachexia, sepsis, metabolic acidosis, spinal injury, systemic infections, denervation, disuse atrophy and the like.

12. A method of screening for an inhibitor of muscle wasting, the method comprising providing a cell-free assay comprising a muscle extract containing components of the N-end rule pathway for protein ubiquitination, exogenous ubiquitin, and a candidate inhibitor; and measuring ubiquitination, wherein a decrease in ubiquitination is indicative of inhibition by said candidate inhibitor.

13. The method of claim 12 wherein the components of the N-end rule pathway for protein ubiquitination comprise an N-end rule ubiquitin conjugating enzyme or an N-terminal protein modifying enzyme.

14. The method of claim 13 wherein the muscle extract is from an atrophying muscle from a mammal afflicted with a condition that results in a catabolic state in muscle tissue of the mammal.

15. The method of claim 14 wherein the mammal exhibits an abnormally high level of muscle wasting.

16. The method of claim 15 wherein the mammal is afflicted with a muscle wasting condition selected from the group consisting of hyperthyroidism, AIDS- or cancer-related cachexia, sepsis, metabolic acidosis, spinal injury, systemic infections, denervation, disuse atrophy and the like.

17. The method of claim 12 wherein the candidate inhibitor is a dipeptide having an amino terminal amino acid which mimics a destabilizing amino terminus of a protein.

18. A method of screening for an inhibitor of muscle wasting, the method comprising providing a cell-free assay comprising a muscle extract containing components of the N-end rule pathway for protein ubiquitination, an ubiquitination substrate, and a candidate inhibitor; and measuring degradation of the substrate, wherein a decrease in substrate degradation is indicative of inhibition by the candidate inhibitor.

19. The method of claim 18 wherein the components of the N-end rule pathway for protein ubiquitination comprise an N-end rule ubiquitin conjugating enzyme or an N-terminal protein modifying enzyme.

20. The method of claim 18 wherein the candidate inhibitor comprises a dipeptide having an amino terminal amino acid which mimics an abnormal amino terminus of a protein.

21. The method of claim 18 wherein the substrate is one of lysozyme or lactalbumin.

22. The method of claim 18 wherein the muscle extract is from an atrophying muscle.

23. The method of claim 22 wherein the atrophying muscle is from a mammal afflicted with a condition that results in a catabolic state in muscle tissue of the mammal.

24. The method of claim 23 wherein the mammal exhibits an abnormally high level of muscle wasting.

25. The method of claim 24 wherein the mammal is afflicted with a muscle wasting condition selected from the group consisting of hyperthyroidism, AIDS- or cancer-related cachexia, sepsis, metabolic acidosis, spinal injury, systemic infections, denervation, disuse atrophy and the like.

26. A kit for screening for a candidate inhibitor of muscle wasting, the kit comprising a component of the N-end rule pathway for protein ubiquitination, exogenous ubiquitin, and packaging materials therefor.

27. The kit of claim 26 wherein the component of the N-end rule pathway for protein ubiquitination comprises an N-end rule ubiquitin conjugating enzyme.

28. The kit of claim 27 wherein the N-end rule ubiquitin conjugating enzyme is an E2 enzyme.

29. The kit of claim 28 wherein the E2 enzyme is $E2_{14K}$.

30. The kit of claim 27 wherein the N-end rule ubiquitin conjugating enzyme comprises an E3 enzyme.

31. The kit of claim 30 wherein the E3 enzyme is E3α.

32. A kit for screening for a candidate inhibitor of muscle wasting, the kit comprising a muscle extract containing a component of the N-end rule pathway for protein ubiquitination, an ubiquitination substrate, and packaging materials therefor.

33. The kit of claim 32 wherein the component of the N-end rule pathway for protein ubiquitination comprises an N-end rule ubiquitin conjugating enzyme.

34. The kit of claim 33 wherein the N-end rule ubiquitin conjugating enzyme is an E2 enzyme.

35. The kit of claim 34 wherein the E2 enzyme is $E2_{14K}$.

36. The kit of claim 33 wherein the N-end rule ubiquitin conjugating enzyme comprises an E3 enzyme.

37. The kit of claim 36 wherein the E3 enzyme is E3α.

38. A method of screening for an inhibitor of muscle wasting, the method comprising providing a cell-based assay comprising muscle cells containing a recombinant DNA construct containing a reporter gene that is responsive to a change in the level of ubiquitin conjugation in the cell via a component of the N-end rule pathway for protein ubiquitination, and a candidate inhibitor; and measuring an increase or decrease in expression of the reporter gene, wherein inhibition is indicated as an increase in expression of the reporter gene.

39. The method of claim 38 wherein the reporter gene is a gene fusion encoding a hybrid protein having an amino terminal amino acid which mimics a destabilizing amino terminus of a protein and a carboxy terminal reporter molecule.

40. The method of claim 39 wherein the reporter molecule comprises β-galactosidase or a detectable portion thereof.

41. The method of claim 40 wherein the hybrid protein amino terminal amino acid is arginine or leucine.

42. A kit for screening for a candidate inhibitor of muscle wasting, the kit comprising a muscle cell containing a recombinant DNA construct comprising a reporter gene that is responsive to a change in the level of ubiquitin conjugation in the cell via a component of the N-end rule pathway for protein ubiquitination, and packaging materials therefor.

43. The kit of claim 42 wherein the reporter gene encodes a hybrid protein having an amino terminal amino acid which mimics a destabilizing amino terminus of a protein and a carboxy terminal reporter molecule.

44. The kit of claim 42 wherein the reporter molecule comprises β-galactosidase or a detectable portion thereof.

45. The kit of claim 43 wherein the hybrid protein amino terminal amino acid is arginine or leucine.

46. A therapeutic composition for use in the treatment of diseases characterized by accelerated muscle wasting, the composition comprising an inhibitor capable of interfering with function of a component of the N-end rule pathway for protein ubiquitination so as to suppress ubiquitination of muscle protein in a pharmaceutically acceptable carrier.

47. The composition of claim 46 wherein the inhibitor is a dipeptide having an amino terminal amino acid which mimics an abnormal amino terminus of a protein.

48. The composition of claim 47 wherein the component of the N-end rule pathway for protein ubiquitination is preferrably an N-end-rule ubiquitin conjugating enzyme.

49. The composition of claim 48 wherein the N-end rule ubiquitin conjugating enzyme is an E2 or E3 enzyme.

50. The composition of claim 49 wherein the E2 enzyme is $E2_{14k}$.

51. The composition of claim 50 wherein the E3 enzyme is E3α.

FIGURE ONE

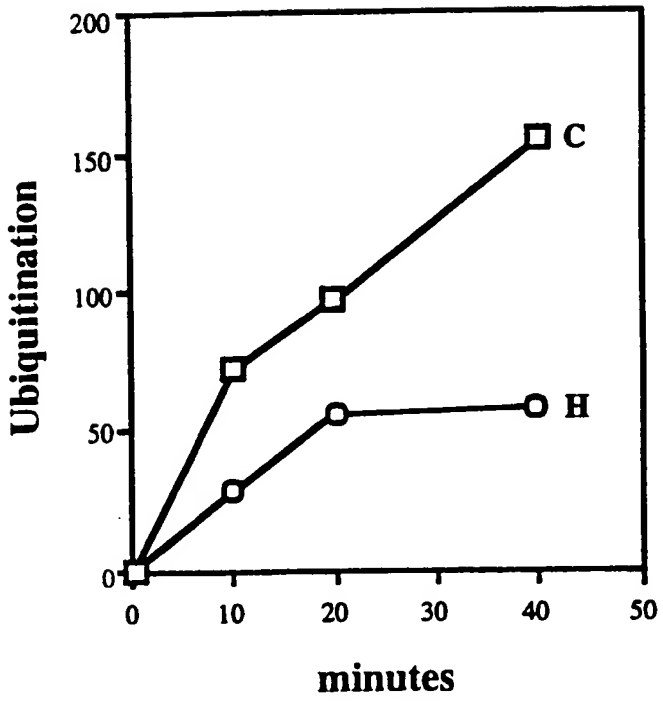


FIGURE TWO

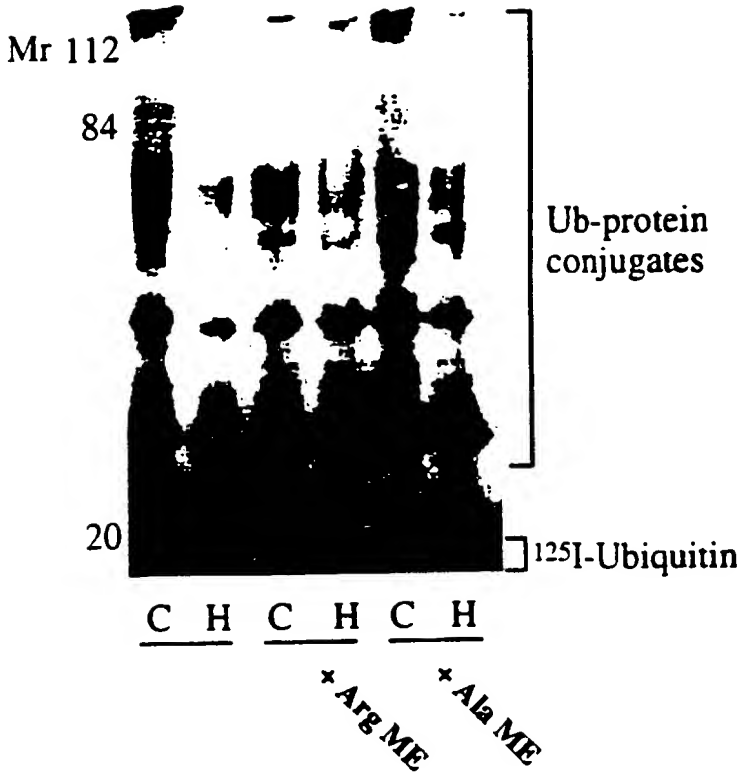

Mr 112
84
20
Ub-protein conjugates
125I-Ubiquitin
C H C H C H
+ Arg ME
+ Ala ME

FIGURE THREE

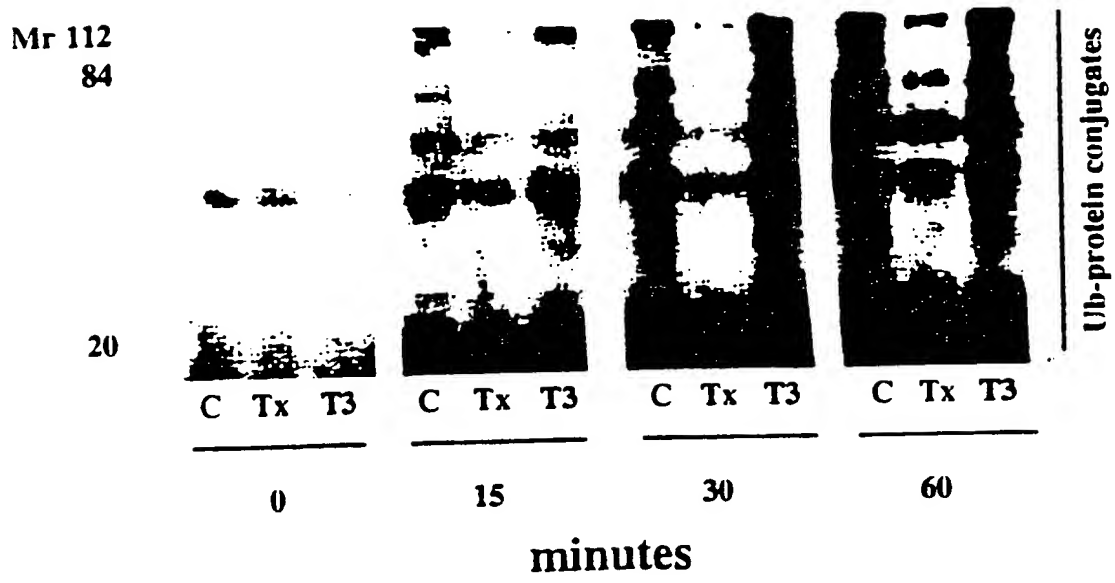


FIGURE FOUR

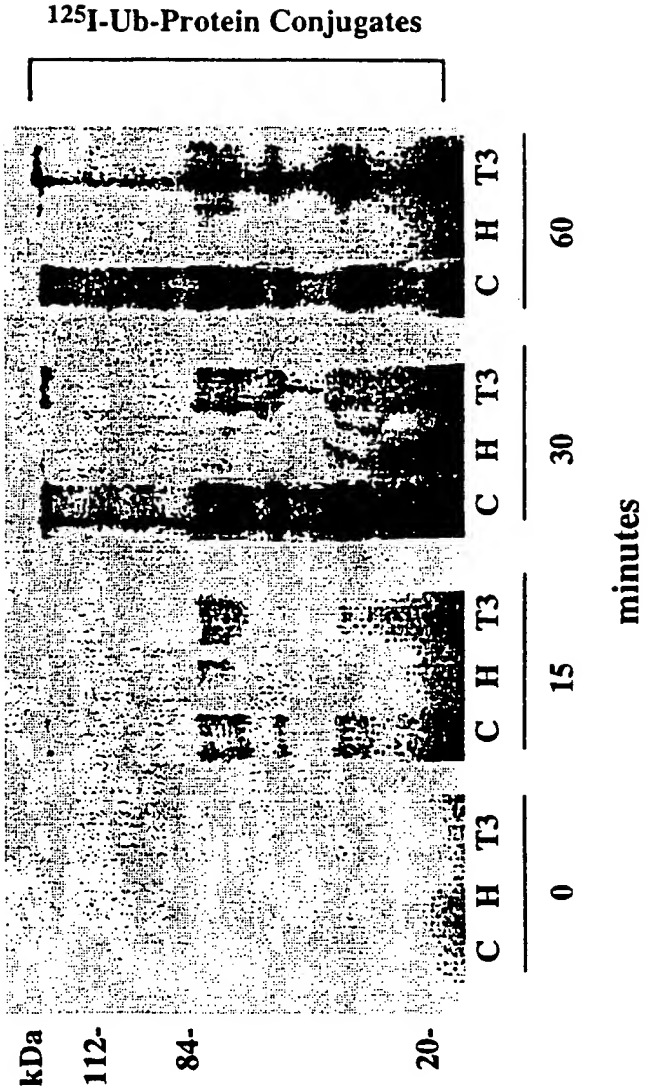

125I-Ub-Protein Conjugates
kDa
112-
84-
20-
C H T3 C H T3 C H T3 C H T3
0
15
30
60
minutes

FIGURE FIVE

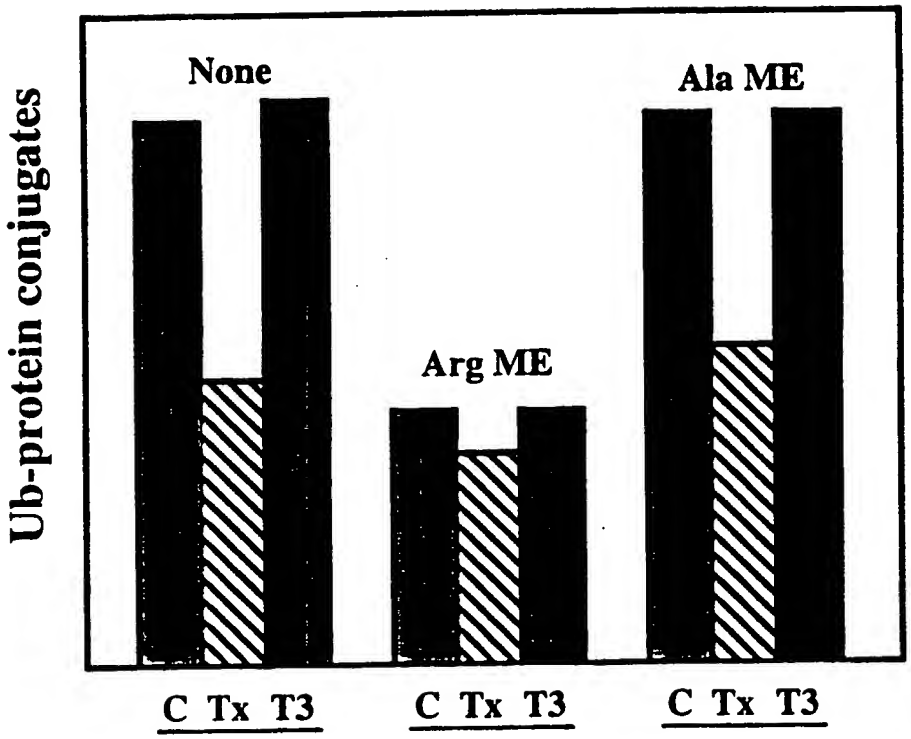


FIGURE SIX

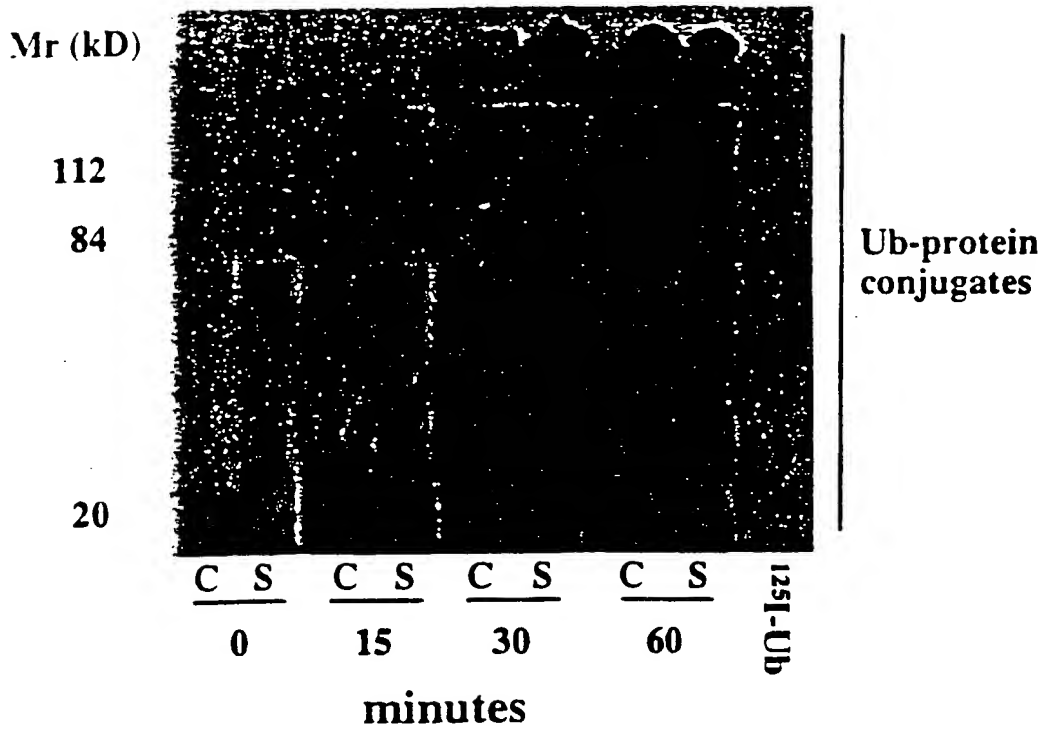

Mr (kD)
112
84
20
Ub-protein conjugates
C S C S C S C S
0 15 30 60
125I-Ub
minutes

FIGURE SEVEN

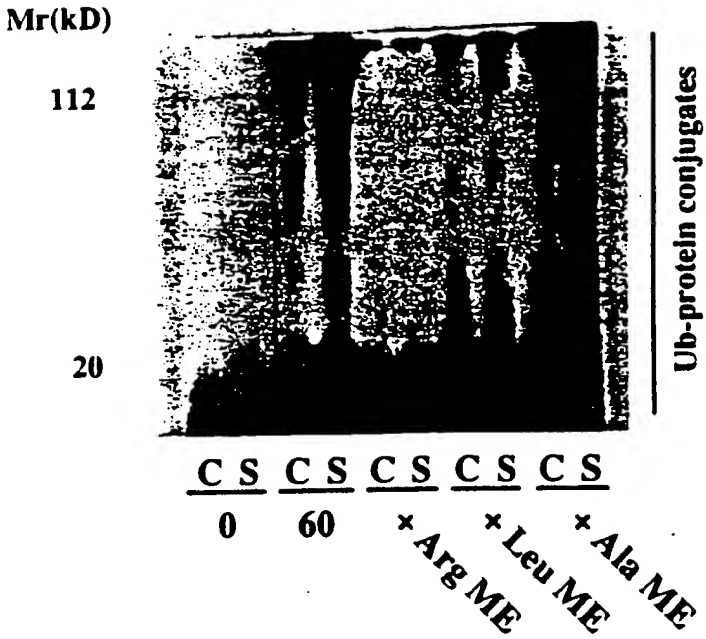


FIGURE EIGHT

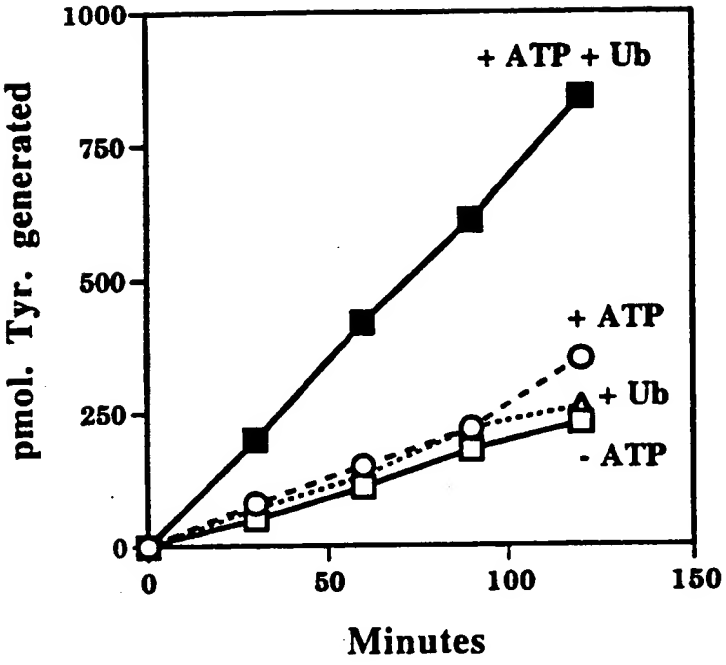


FIGURE NINE

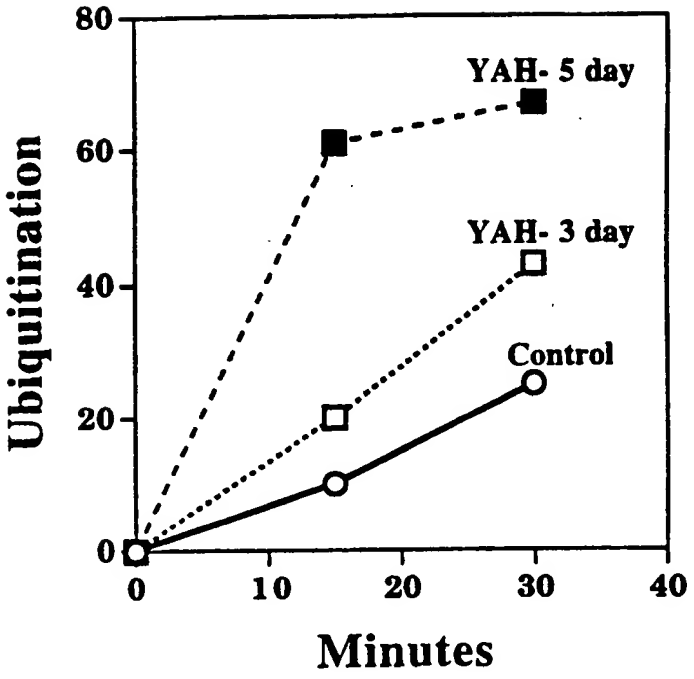

80
60
40
20
0
Ubiquitination
YAH- 5 day
YAH- 3 day
Control
0
10
20
30
40
Minutes

FIGURE TEN

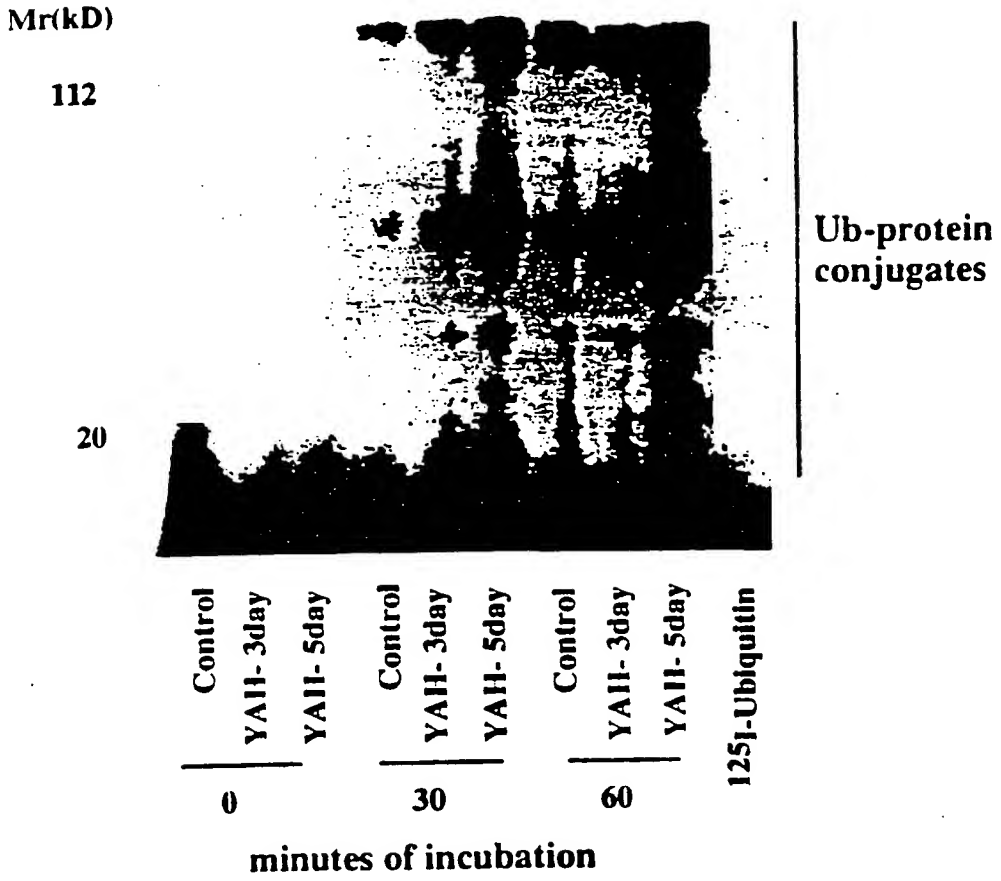

Mr(kD)
112
20
Ub-protein conjugates
Control
YAH-3day
YAH-5day
Control
YAH-3day
YAH-5day
Control
YAH-3day
YAH-5day
125I-Ubiquitin
0
30
60
minutes of incubation

FIGURE ELEVEN

^{125}I-Ubiquitin Conjugation to Endogenous Soluble Proteins in Muscle Extracts from Control Mice, Mice Bearing Colon-26 Tumor and Mice after Tumor removal

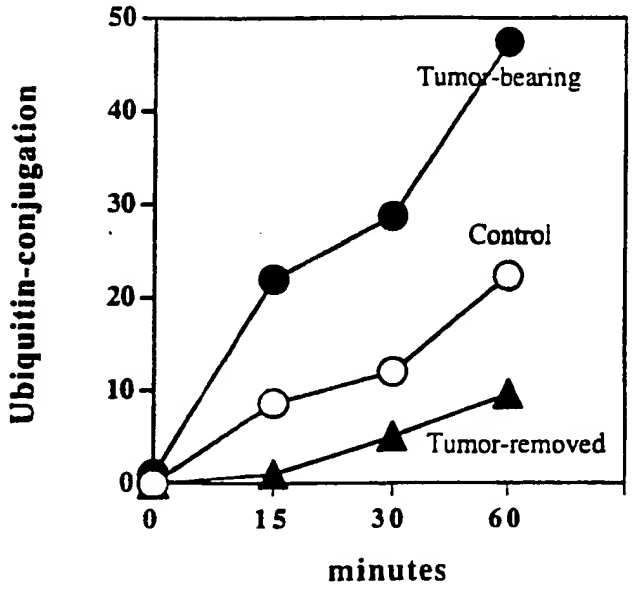


FIGURE TWELVE

Ubiquitin Conjugation to ^{125}I-Lysozyme in Muscle Extracts from Control Mice, Mice bearing Colon-26 Tumor and Tumor-Removed Mice

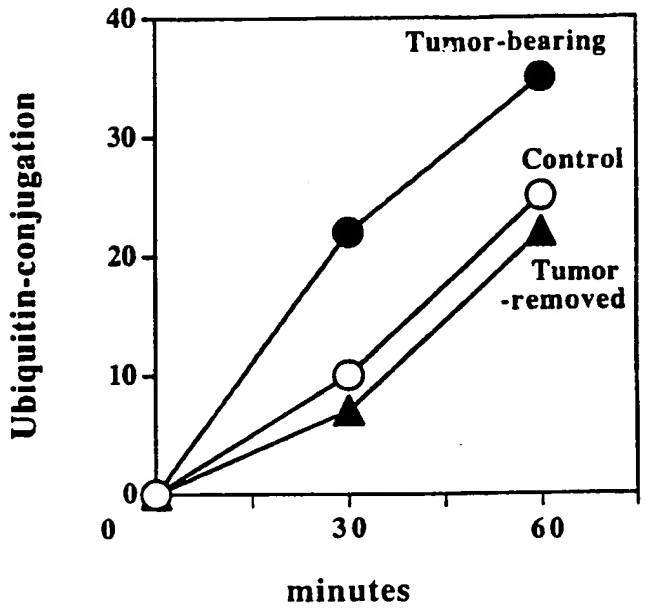


FIGURE THIRTEEN

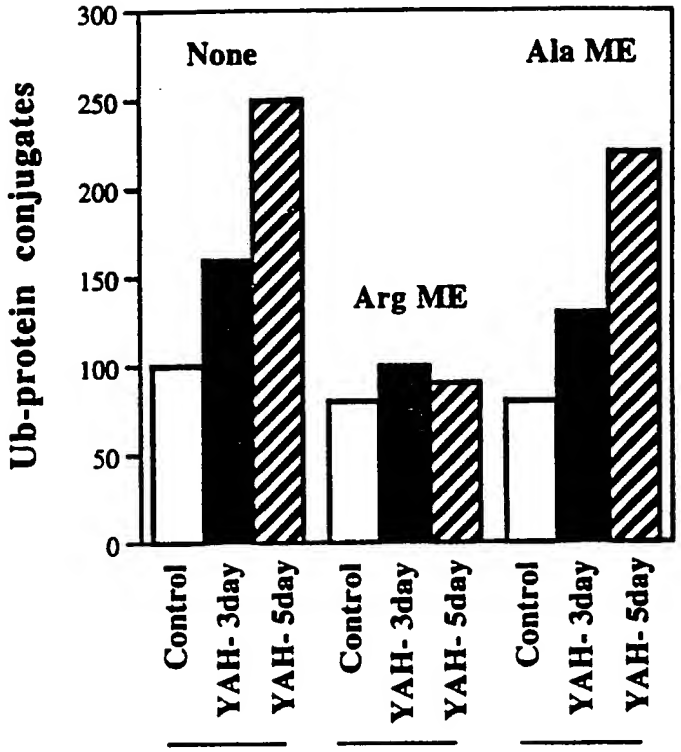


FIGURE FOURTEEN

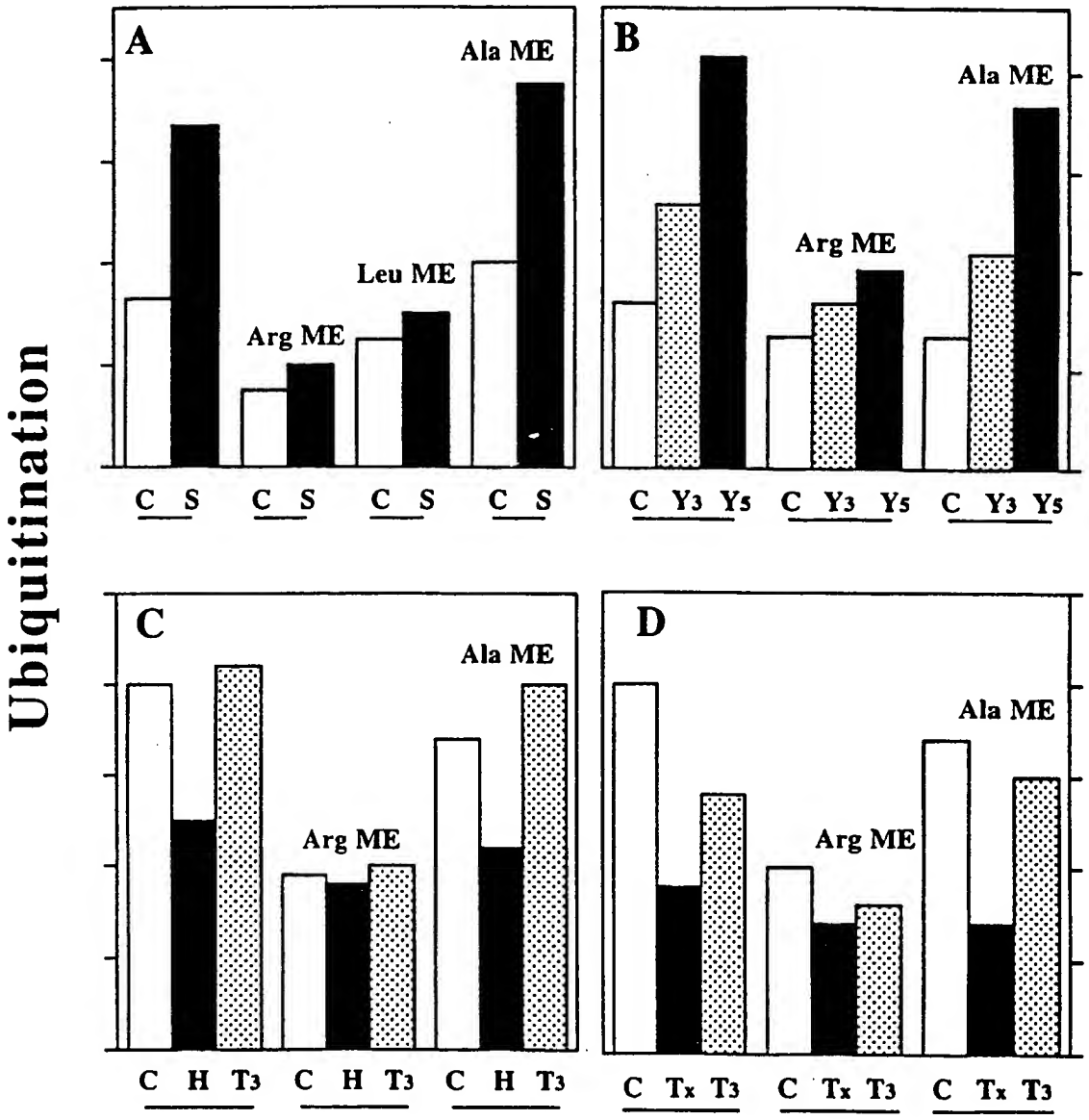


FIGURE FIFTEEN

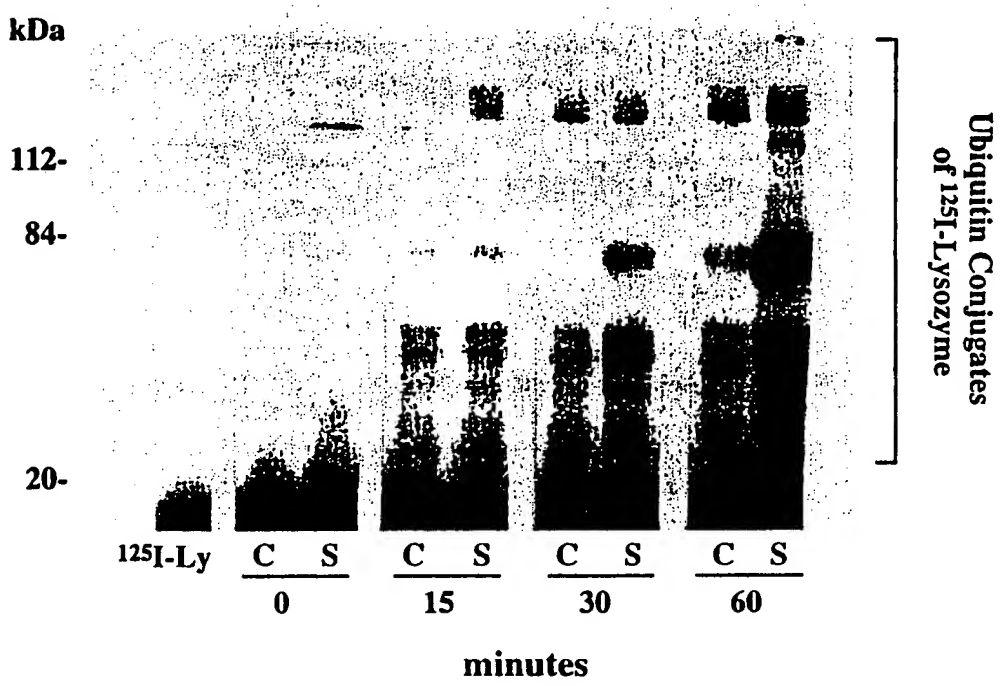


FIGURE SIXTEEN

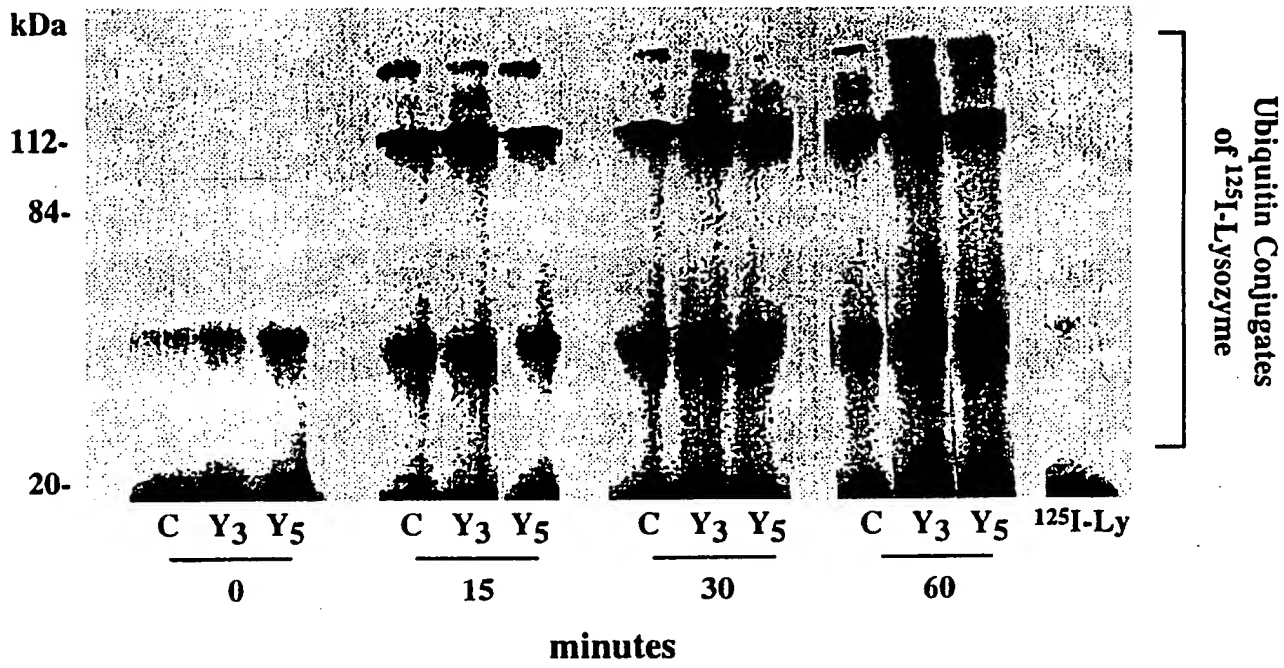
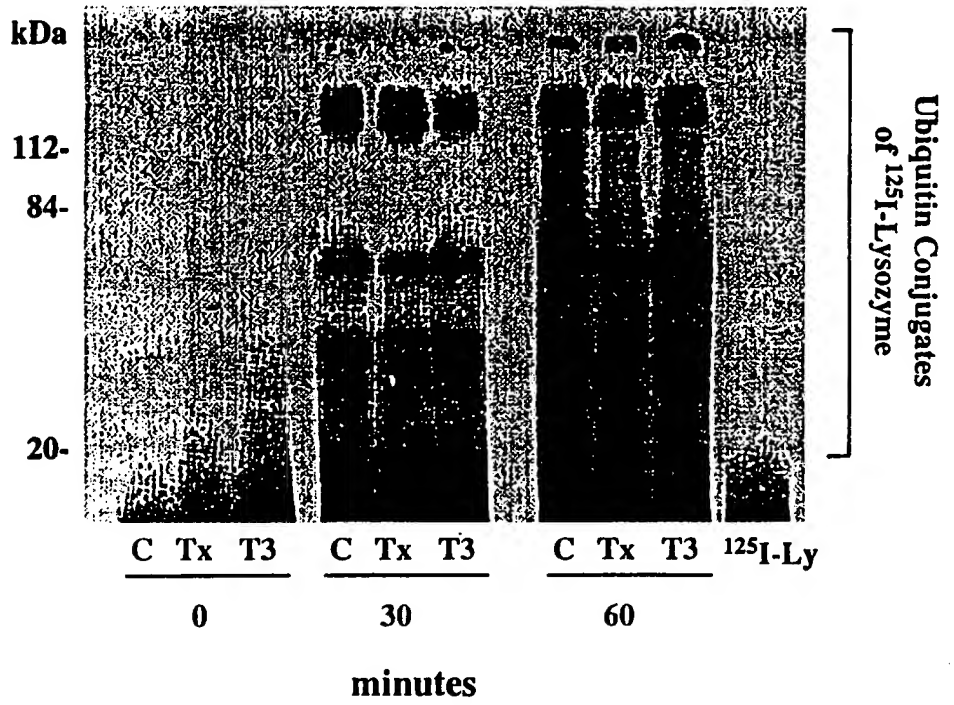


FIGURE SEVENTEEN



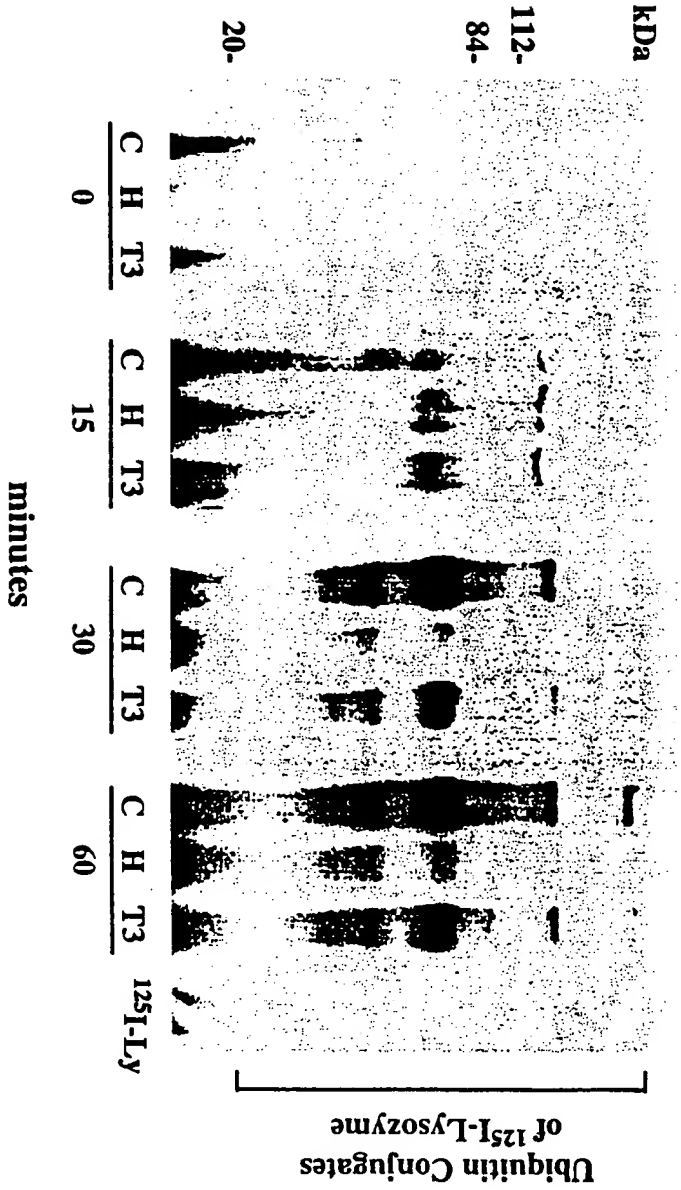


FIGURE EIGHTEEN

FIGURE NINETEEN

^{125}I-Ubiquitin conjugation to soluble muscle proteins changes in pathologic states

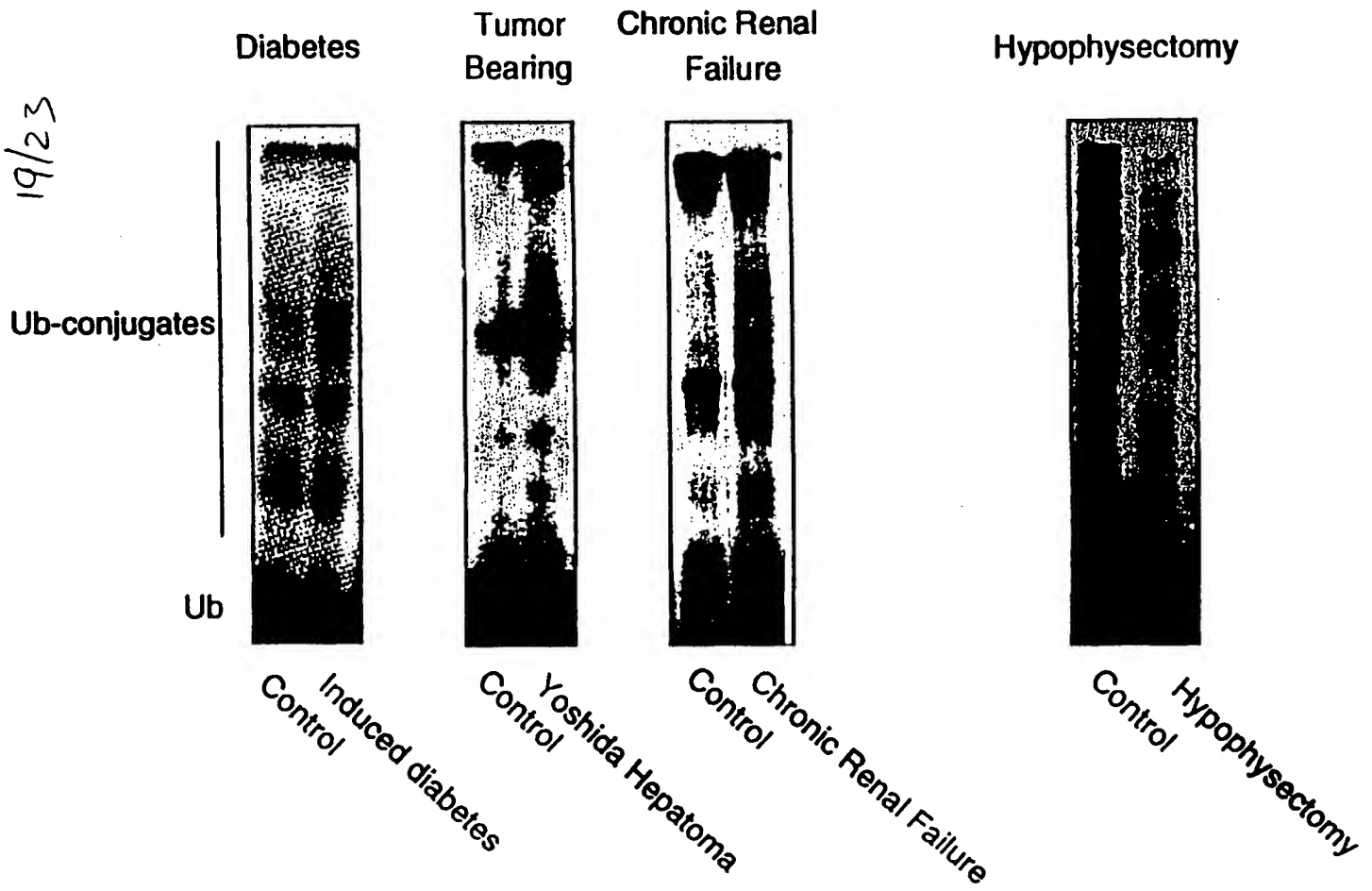
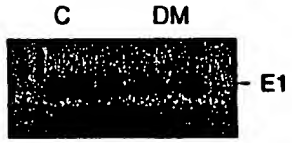


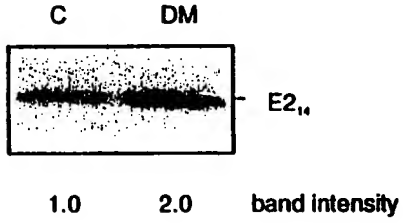
FIGURE TWENTY

Streptozotocin-Induced Diabetes

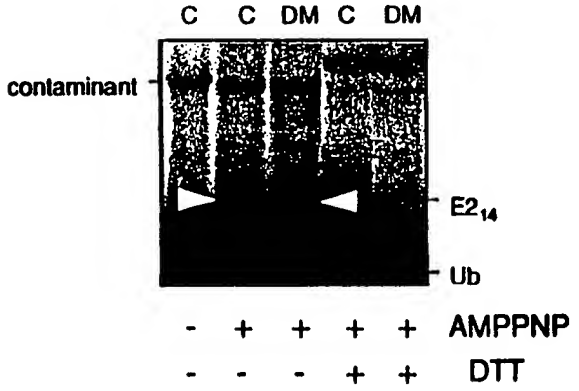
E1: Anti-E1 Western



E2s: Anti-$E2_{14}$ Western



Thiolester assay

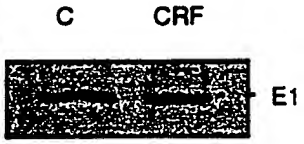


Conclusions: In muscles from rats rendered diabetic, both $E2_{14}$ amount and activity increases, while E1 levels do not change.

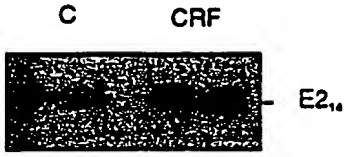
FIGURE TWENTY-ONE

Chronic Renal Failure

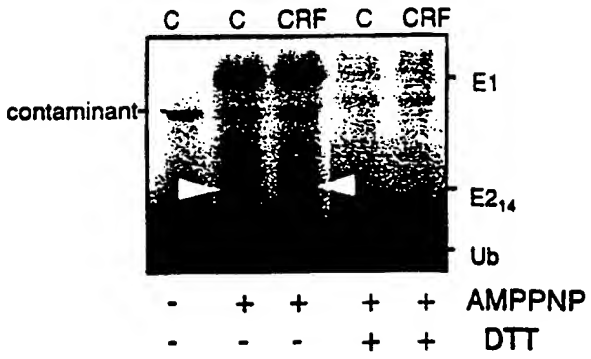
E1: Anti-E1 Western



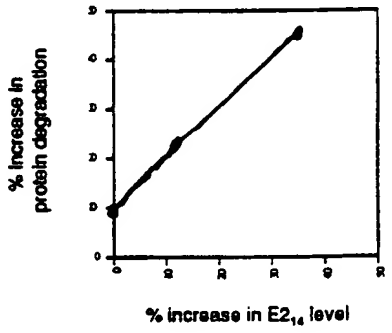
E2s: Anti-$E2_{14}$ Western



ThiolesterAssay



changes in protein degradation correlate with changes in $E2_{14}$ levels in 4 pairs of individual muscles from control/chronic renal failure rats

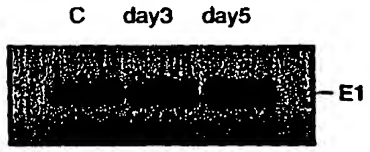


Conclusion: In rats with chronic renal failure, muscles show a trend towards increased levels and activity of $E2_{14}$.

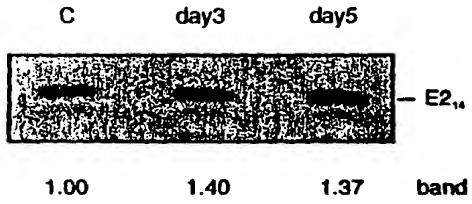
FIGURE TWENTY-TWO

Yoshida Ascities Hepatoma

E1: Anti-E1 Western



E2: Anti-$E2_{14}$ Western

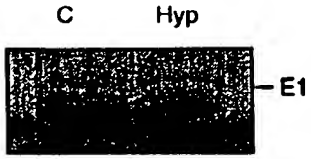


Conclusions: In muscles from rats bearing tumors, levels of $E2_{14}$ rise while levels of E1 are unchanged.

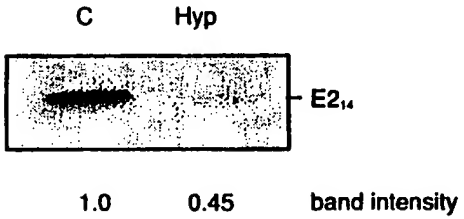
FIGURE TWENTY-THREE

Hypophysectomy

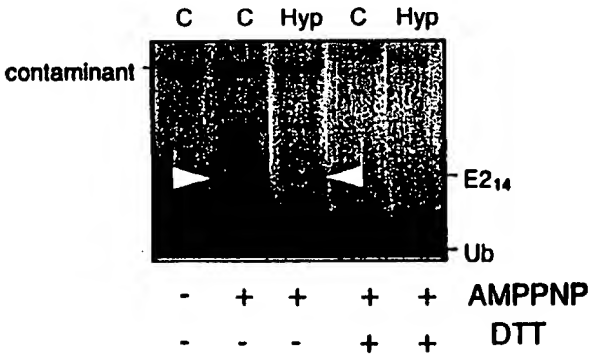
E1: Anti-E1 Western



E2s: Anti-$E2_{14}$ Western



Thiolester assay



Conclusions: In muscles from hypophysectomized rats, where protein breakdown is reduced below normal, the level of $E2_{14}$ and its activity fall while E1 levels do not change.

INTERNATIONAL SEARCH REPORT

Interr ial Application No
PCT/US 97/21421

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K38/05 A61K31/215 C12Q1/25 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 24914 A (MYOGENICS) 21 September 1995 see the whole document ---	1-11, 46-51
A	WO 92 20804 A (PRESIDENT AND FELLOW OF HARVARD COLLEGE) 26 November 1992 cited in the application see the whole document ---	1-51
A	WING S S ET AL: "Increase in ubiquitin-protein conjugates concomitant with the increase in proteolysis in rat skeletal muscle during starvation and atrophy denervation." BIOCHEMICAL JOURNAL, (1995 MAY 1) 307 (PT 3) 639-45, XP002060612 cited in the application see the whole document --- -/--	1-51

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

3

Date of the actual completion of the international search	Date of mailing of the international search report
30 March 1998	22.04.1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Moreau, J

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	ROCK K L ET AL: "Inhibitors of the proteasome block the degradation of most cell proteins and the generation of peptides presented on MHC class I molecules." CELL, (1994 SEP 9) 78 (5) 761-71, XP002060613 see the whole document ---	1-51
A	CIECHANOVER A.: "The Ubiquitin-Proteasome Proteolytic Pathway" CELL, vol. 79, 7 October 1994, pages 13-21, XP002060614 cited in the application see the whole document -----	1-51

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US 97/21421

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Remark : Although claims 1-11 are directed to a method of treatment of the human/animal body , the search has been carried out and based on the alleged effects of the compound/composition.

INTERNATIONAL SEARCH REPORT

Ir. ational application No.
PCT/US 97/21421

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

see FURTHER INFORMATION sheet PCT/ISA/210

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

...formation on patent family members

Inter ...al Application No
PCT/US 97/21421

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9524914 A	21-09-95	AU 682600 B	09-10-97
		AU 2228395 A	03-10-95
		CA 2185326 A	21-09-95
		EP 0804216 A	05-11-97
		FI 963602 A	04-11-96
		JP 9511501 T	18-11-97
		NO 963814 A	14-11-96
		US 5693617 A	02-12-97
WO 9220804 A	26-11-92	US 5340736 A	23-08-94
		AU 666854 B	29-02-96
		AU 2027592 A	30-12-92
		CA 2102195 A	14-11-92
		EP 0591316 A	13-04-94
		HU 66512 A	28-11-94
		JP 6507786 T	08-09-94
		US 5565351 A	15-10-96